Functional characterization of olfactory receptors in the Oriental fruit fly Bactrocera dorsalis that respond to plant volatiles

AUTHOR(S):

Miyazaki, Hitomi; Otake, Jun; Mitsuno, Hidefumi; Ozaki, Katsuhisa; Kanzaki, Ryohei; Chui-Ting Chieng, Anna; Kah-Wei Hee, Alvin; Nishida, Ritsuo; Ono, Hajime

Functional characterization of olfactory receptors in the Oriental fruit fly *Bactrocera dorsalis* that respond to plant volatiles

Hitomi Miyazaki[a,1], Jun Otake[a], Hidefumi Mitsuno[b], Katsuhisa Ozaki[c], Ryohei Kanzaki[b], Anna Chui-Ting Chieng[d], Alvin Kah-Wei Hee[d], Ritsuo Nishida[a], Hajime Ono[a,1,*]

[a]Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

[b]Research Center for Advanced Science and Technology, University of Tokyo, Tokyo 153-8904, Japan

[c]JT Biohistory Research Hall, Takatsuki Osaka, 569-1125, Japan

[d]Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia

*Corresponding author. Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwakecho, Sakyo-ku, Kyoto, 606-8502, Japan

E-mail address: onoono@kais.kyoto-u.ac.jp

[1]These authors contributed equally to this work

ABSTRACT

The Oriental fruit fly, *Bactrocera dorsalis*, is a highly destructive pest of various fruits. The reproductive and host-finding behaviors of this species are affected by several plant semiochemicals that are perceived through chemosensory receptors. However, the chemosensory mechanisms by which this perception occurs have not been fully elucidated. We conducted RNA sequencing analysis of the chemosensory organs of *B. dorsalis* to identify the genes coding for chemosensory receptors. We identified 60 olfactory receptors (ORs), 17 gustatory receptors and 23 ionotropic receptors—including their homologs and variants—from the transcriptome of male antennae and proboscises. We functionally analyzed ten ORs co-expressed with the obligatory co-receptor ORCO in *Xenopus* oocytes to identify their ligands. We tested 24 compounds including attractants for several *Bactrocera* species and volatiles from the host fruits of *B. dorsalis*. We found that BdorOR13a co-expressed with ORCO responded robustly to 1-octen-3-ol. BdorOR82a co-expressed with ORCO responded significantly to geranyl acetate, but responded weakly to farnesenes (a mixture of isomers) and linalyl acetate. These four compounds were subsequently subjected to behavioral bioassays. When each of the aforementioned compound was presented in combination with a sphere model as a visual cue to adult flies, 1-octen-3-ol, geranyl acetate, and farnesenes significantly enhanced landing behavior in mated females, but not in unmated females or males. These results suggest that the ORs characterized in the present study are involved in the perception of plant volatiles that affect host-finding behavior in *B. dorsalis*.

Keywords: Chemosensory receptor; *Bactrocera dorsalis*; Plant semiochemical; Functional analysis; *Xenopus* oocyte; Behavioral bioassay

1. Introduction

Plant semiochemicals play a crucial role in insect–plant interactions, because they affect insect physiology and behavior (Reddy and Guerrero, 2004). Many phytophagous insects use plant semiochemicals as cues to find their feeding, mating, and oviposition sites. Moreover, some insects specifically recognize host plant chemicals via chemosensory organs to acquire or sequester those chemicals as defensive substances, sex pheromones, or sex pheromone precursors (Nishida, 2002; Opitz and Müller, 2009). Therefore, an elucidation of the basic mechanisms underlying the chemoreception of plant semiochemicals is essential for an understanding of the adaptation of phytophagous insects to plants as sources of essential substances for growth and reproduction.

The tephritid fruit fly species, which include destructive horticultural pests in both tropical and temperate regions, provide a good model for understanding how insects adapt to plant chemicals, because their life cycles involve response to several characteristic semiochemicals (Metcalf, 1990; Shelly, 2010). Several such species belonging to two Dacinae genera, *Bactrocera* and *Zeugodacus*, exhibit striking behaviors towards certain semiochemicals that contribute to the floral fragrances of several orchid species. For example, male Oriental fruit flies (*Bactrocera dorsalis*) are strongly attracted to a specific phenylpropanoid, methyl eugenol (ME). This leads to voracious consumption of the compound by the male flies, and its subsequent utilization as a sex pheromone precursor (Howlett, 1912; Nishida et al., 1988; Tan and Nishida, 2012). Furthermore, volatiles derived from host fruits play a crucial role in the search for oviposition sites by gravid females. Electrophysiological studies using gas chromatography-flame ionization detection coupled with electroantennographic

detection (GC-EAD) have shown that a number of compounds including terpenes and phenylpropanoids derived from host fruits elicit female antennal responses in *B. dorsalis* (Siderhurst and Jang, 2006a; Siderhurst and Jang, 2006b; Kamala Jayanthi et al., 2012; Kamala Jayanthi et al., 2014; Damodaram et al., 2014).

Although the perception of plant semiochemicals is so important in the diverse life history of tephritid fruit flies, as described above, the mechanisms by which chemoreception occurs have not been fully elucidated. The major molecular components of insect chemoreception have been identified mainly from studies on *Drosophila melanogaster* (Fleischer et al., 2017); chemosensory receptors are essential for the recognition of ligands at peripheral neurons. Insect chemosensory receptors consist of three types of insect-specific superfamilies: olfactory receptors (ORs), gustatory receptors (GRs), and ionotropic receptors (IRs) (Vosshall and Stocker, 2007; Touhara and Vosshall, 2009; Rytz et al., 2013). These receptors are thought to form ligand-gated ion channels and/or to function as G-protein-coupled receptors (GPCRs). Among these insect receptor superfamilies, ORs have been relatively well characterized as heteromeric ligand-gated ion channels that consist of a specific OR and a highly conserved co-receptor ORCO (Sato et al., 2008; Wicher et al., 2008). Because a specific odorous ligand is tuned to a specific OR in this system, the identification of the ligands for uncharacterized ORs could provide clues to the essential chemicals involved in the life cycle of *Bactrocera* species.

A previous study has shown that the odorant receptor co-receptor, ORCO, is involved in the perception of ME in *B. dorsalis*, suggesting that specific ORs are required for the chemoreception of ME and its metabolites (Zheng et al., 2012). Furthermore, chemosensory genes that code for ORs, IRs, and GRs have been identified in the transcriptome of *B. dorsalis* (Wu et al., 2015; Liu et al., 2016).

Importantly, it is possible to access the reference genome sequences of *B. dorsalis* at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/genome/10754). Although the sequence data for *B. dorsalis* has been obtained, no chemosensory receptors responding to semiochemicals have been characterized. In the present study, we conducted RNA sequencing (RNA-seq) analysis of the chemosensory organs of *B. dorsalis* to identify genes coding for chemosensory receptors. We identified 60 ORs, 17 GRs, and 23 IRs—including their homologs and variants—from the transcriptome of male antennae and proboscises. We characterized the functional properties of two ORs that respond to plant semiochemicals in a heterologous expression system comprising *Xenopus* oocytes. We further assessed the attraction of both female and male flies to four volatiles recognized as ligands for the two ORs, and found that when used in combination with visual cues, certain plant volatiles had a significant effect on the landing behavior of mated females. In the present study, we demonstrated the functional identification of specific ligands for chemosensory receptors, which should provide clues to the identity of chemicals that influence insect behaviors.

2. Materials and methods

2.1. Insects

For preparation of total RNA, we obtained a strain of *B. dorsalis* from a colony maintained by the Naha Plant Protection Station in Okinawa, Japan. The strain—originating in Okinawa, Japan—was reared with the permission of the Minister of Agriculture, Forestry, and Fisheries of Japan (permit No. 56Y-1882). For the behavioral bioassay, we used a laboratory-reared colony of *B. dorsalis* from the

Department of Biology, the Faculty of Science, Universiti Putra Malaysia. The flies were kept at 25–29°C and 83–90% relative humidity, and subjected to a 12-h light/12-h dark photoperiod regimen. The adult flies were given *ad libitum* access to water and a mixture of sugar and hydrolyzed protein (3:1 w/w). Males and females were separated within 3 days of emergence to prevent mating, and kept in cages (30 cm × 30 cm × 30 cm) until required for the bioassay, which took place 16–19 days after emergence. Mated flies were obtained in the following manner. Two days before the bioassay, virgin males and females were placed together in a cage in the morning. The mated pairs (> 30 min of non-stop copulation) were gently collected in the late evening using a glass vial (2 mm diameter × 8.5 cm), and placed in a separate cage containing food and water. This was done to ensure that the copulating pairs did not separate from each other. Those flies were then segregated again by sex into separate cages (40 cm × 40 cm × 40 cm), and allowed to acclimatize in a sheltered outdoor bioassay area that received sunlight from the east before the experiment commenced the next morning. The virgin, males and females were also separated and allowed to acclimatize in the bioassay area prior to the behavioral trials.

2.2. RNA sequencing and assembly

The male flies were staged at 0–2, 2–4, 3–5 and 5–7 days after eclosion. Approximately 150 males were collected from each adult stage. Their antennae and proboscises were dissected and homogenized in TRIzol Reagent (GIBCO-BRL, Gaithersburg, MD, USA). Total RNAs were extracted from the homogenates and purified using NucleoSpin RNA (Macherey-Nagel, Germany). Sequence libraries were prepared using the TruSeq RNA Sample Preparation Kit v2 (Illumina, Inc., San Diego, CA, USA) as described previously (Yang et al., 2015). RNA sequencing was

performed on an Illumina MiSeq system using the MiSeq Reagent Kit v3 600 cycle (Illumina, Inc., San Diego, CA, USA). The reads were preprocessed with Trimmomatic v0.33 (Bolger et al., 2014) for quality trimming using the following parameters: LEADING: 10; TRAILING: 10; SLIDINGWINDOW: 4:20; MINILEN: 150. The resulting clean reads data have been deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive under accession number PRJDB6798. The pass-through reads were subjected to *de novo* assembling using the Trinity, Bowtie, eXpress, and DEGseq (PE) programs (Grabherr et al., 2011) implemented in the maser pipeline of the Cell Innovation Program at the National Institute of Genetics (http://cell-innovation.nig.ac.jp/index_en.html). Fragments per kilobase of exon per million (FPKM) values were calculated to estimate the expression levels of the transcripts.

2.3. Screening and characterization of sequences of candidate chemosensory receptors

We identified candidate chemosensory receptor genes from the Trinity contigs using the Pfam database. For this purpose, we obtained four protein domain families of *D. melanogaster* from the Pfam database (http://pfam.xfam.org): 7tm odorant receptor (PF02949), 7tm chemosensory receptor (PF08395), trehalose receptor (PF06151), and ligand gated ion channel (PF00060). We screened the Trinity contigs by similarity to these protein domain families using a BLASTX search at an Expect value (E-value) threshold of 1e-5. In parallel, we analyzed the Trinity contigs using a TBLASTN search against protein databases consisting of chemosensory receptors of *D. melanogaster* at the same E-value threshold. We obtained open reading frames (ORFs) of the extracted contigs using EMBOSS Transeq

(https://www.ebi.ac.uk/Tools/st/emboss_transeq/), and used them as queries in a BLASTP search against the NCBI non-redundant protein database. Contigs that ranked highly with ORs, GRs, or IRs were considered candidate genes coding for insect chemosensory receptors. Overlapping variants with identical ORFs were merged at this step by selecting the longest as the representative transcript of a variant group. Full-length ORFs of several ORs were predicted from genome sequences in the genome sequencing and assembly project, and were cloned into a vector, as described in Section 2.4. Candidate chemosensory receptors were named according to the following criteria. i) Chemosensory receptors were named as described in a previous paper (Wu et al., 2015) when their amino acid sequences were identical. ii) Orthologs of chemosensory receptors uncharacterized from *B. dorsalis* were named according to those of *D. melanogaster*. iii) For homologous chemosensory receptors with amino acid similarities of less than 80%, the names of the homologs were differentiated with a numerical postscript, e.g., *BdorOR7a-1* and *BdorOR7a-2*. iv) In cases where the amino acid similarities were 80% or more, version numbers were assigned to the receptors, e.g., *BdorOR67c-v1* and *BdorOR67c-v2*. v) In cases where multiple partial sequences of a candidate chemosensory receptor were identified, each sequence was labeled *-part1*, *-part2*, etc., e.g., *BdorOR2a-part1* and *BdorOR2a-part2*. To compare amino acid sequences of chemosensory receptors identified from *B. dorsalis* between the present study and previous studies, we performed BLASTP searches with an E-value cutoff of 1e-100.

2.4. Cloning of full-length coding sequences of candidate ORs into an expression vector

We cloned full-length coding sequences of candidate ORs into a pCS2P+ vector kindly provided by Prof. Marc Kirschner (https://www.addgene.org/17095/). The primers were designed from the predicted ORFs based on the assembled contigs or reference genome sequences of *B. dorsalis* at the NCBI web site (https://www.ncbi.nlm.nih.gov/genome/?term=JFBF01). The ORFs of *BdorORCO*, *BdorOR94b-1*, and *BlatOR59a* were amplified by PCR from cDNA prepared from male antennae using primers including untranslated regions based on the contig sequences. The PCR products were then cloned into a pGEM-T vector (Promega, WI, USA). The ORFs were modified with a Kozak consensus sequence (5′-GCCGCC-3′) and the appropriate restriction site by PCR amplification using the following primers. The forward primer included the Kozak consensus sequence followed by a BamHI restriction site, and the reverse primer included an XbaI restriction site. The PCR products were cloned into a pCS2P+ vector using the restriction sites. The ORFs of the other ORs were amplified by PCR as described above, except that the primers included a part of the pCS2P+ sequence to enable cloning into the vector using an In-Fusion HD cloning Kit (Takara, Otsu, Japan). The PCR reactions were performed using AmpliTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The primers used for construction are listed in Table S1.

2.5. Phylogenetic analysis

Deduced amino acid sequences of candidate ORs were aligned using the Clustal W 2.1. program (Thompson et al., 1994). Prior to this process, we merged the partial sequences of *BdorOR2a*, *BdorOR7a*-8, *BdorOR24a*, *BdorOR45a*, and *BdorOR63a1-v1*. We selected candidate ORs with sequences of more than 150 amino acid for

phylogenetic analysis, and constructed a phylogenetic tree from the aligned sequences. We applied the maximum likelihood method with the Jones–Taylor–Thornton (JTT) model with among-site rate heterogeneity according to gamma distribution with invariant sites (G + I) using MEGA5 software (Tamura et al., 2011). We performed 1000 bootstrap replicates.

2.6. Expression analyses of the candidate receptors by RT-PCR and quantitative RT-PCR (qPCR)

Total RNAs were prepared from various tissues of the staged adults within 2 days of eclosion, as described above. Reverse transcription was performed using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Tsuruga, Japan). The generated cDNAs were subjected to PCR amplification with gene-specific primers using the GoTaq Green Master Mix (Promega, WI, USA). The PCR conditions were: 94°C for 1 min; and 35 or 40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and 72°C for 2 min. Alternatively, the cDNA were used as a template for qPCR using the THUNDERBIRD SYBR qPCR Mix (TOYOBO, Tsuruga, Japan) on a Thermal Cycler Dice Real Time System (Takara, Shiga, Japan). We investigated five or six independent biological samples to quantify the levels of transcription. The transcription levels were normalized with *rpS3* transcription levels in the same samples. The primers used for RT-PCR and qPCR are listed in Table S2.

2.7. Chemicals

The chemicals used for the functional analysis of the BdorORs are listed in Table S3, and their structures are shown in Fig. S1. We synthesized 3-oxo-7,8-dihydro-α-ionone (P3) according to the method described in a previous paper (Enomoto et al., 2010).

We synthesized 4-propionyloxyisophorone (E0P) from 4-oxoisophorone (TCI, Tokyo, Japan) (Nishida and Tan, 2016). Briefly, the carbonyl function of 4-oxoisophorone at C-1 was protected by converting it into a ketal group using ethylene glycol. The carbonyl function at C-4 was then reduced to a hydroxyl moiety, and the ketal at C-1 was simultaneously deprotected using $NaBH_4$. The product was then propionylated into E0P using anhydrous propionic acid.

2.8. Receptor expression in Xenopus *oocytes and two-electrode voltage-clamp recording*

The preparation of *Xenopus laevis* oocytes, the microinjection of receptor gene RNAs, and the recording of whole-cell currents were performed as described previously with minor modifications (Mitsuno et al., 2008). In brief, complementary RNAs (cRNAs) were synthesized from linearized pCS2P+ vectors containing the full-length coding sequences of the ORs using a mMESSAGE mMACHINE T7 Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). Stage V to VII *Xenopus* oocytes treated with collagenase in Ca^{2+}-free saline solution were microinjected with a mixture comprising *OR* and *BdorORCO* cRNAs (2.5 ng each). Using a two-electrode voltage clamp (OC-725, Warner, Hamden, CT, USA), we recorded whole cell currents from injected oocytes after incubation for 5–7 days at 20°C in an assay buffer comprising 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1.6 mM $MgCl_2$, 2.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 2.5 mM 2-(*N*-morpholino)ethanesulfonic acid MES (pH 7.5). The inward current was monitored at a holding potential of −80 mV. Each ligand was diluted with the assay buffer to a specific concentration containing 0.1% dimethyl sulfoxide (DMSO). The assay buffer containing 0.1% DMSO was used as a negative control. Data acquisition and analyses

were carried out using Digidata 1322A and pCLAMP software (Axon Instruments, Foster City, CA, USA).

2.9. Behavioral bioassays

We evaluated the attraction of the flies to 1-octen-3-ol, geranyl acetate, farnesenes, and linalyl acetate, in combination with white or green sphere models as visual cues. Ethanol was used as a control. We used a total of four spheres (one green with a test compound and one green with ethanol; one white with a test compound and one white with ethanol) for attraction in each of the four groups of flies (Fig. S2A). Sexually mature adult flies (50 virgin females, 50 virgin males, 50 mated females, or 50 mated males) were transferred to a meshed cage (40 cm × 40 cm × 40 cm; placed in a sheltered outdoor bioassay area) in the morning 1 day prior to commencement of the experiment for acclimatization. On the day of the experiment (08:00–11:00), we impregnated pieces of Whatman® No. 1 filter paper (15 mm × 3 mm) with 1 mg of each of the test compounds dissolved in 5 μL of ethanol, and dried them at room temperature. Each filter paper was then placed in a clean 0.2-mL clear microtube (with the cap removed) (Labchem, Malaysia), which was positioned facing up in one of the holes of a polyethylene sphere that consisted of 26 holes (sphere diameter 40 mm; hole diameter 6 mm; Catalog No. GV0310, Tabata Co., Ltd., Tokyo, Japan). Each sphere was placed on a plastic petri dish (diameter 5 cm) to prevent rolling on the cage floor during the bioassay (Fig. S2B). Ethanol was used as a control. Each of the four spheres was then placed 10 cm from its respective corner in the meshed cage. The position of the spheres based on color and compound combination was re-randomized in each of the 4–6 replicates used, with different cohorts of flies tested

297 each time. Fruit flies landing on the spheres were counted and rapidly removed by
298 aspiration during the 15-min bioassay.
299
300 ***2.10. Statistical analysis***
301 Statistical analyses were conducted using R software (www.r-project.org). Dose
302 responses were analyzed using the four-parameter log-logistic model of the *drc*
303 extension package (Ritz et al., 2015). For the behavioral bioassay, we used a
304 generalized linear model (GLM) with binomial distribution to determine whether the
305 volatile compound or the color of the sphere significantly affected the number of fruit
306 flies landing on the sphere. The most parsimonious model was identified using the
307 Akaike information criterion (AIC). The likelihood ratio test (LRT) with chi-square
308 distribution was used to determine the difference between the nested models.
309
310 **3. Results**
311
312 ***3.1. RNA sequencing and identification of chemosensory receptors***
313 We obtained 1,675,116 and 2,159,685 raw reads from the transcriptomes of the male
314 antennae and proboscises, respectively, using the Illumina MiSeq system (Table 1).
315 After removing the low-quality, adaptor, and contaminating sequence reads, the male
316 antennae and proboscises yielded 1,159,879 and 1,383,389 clean reads, respectively,
317 which were assembled into 71,766 contigs (S1 text). We identified chemosensory
318 receptors—namely, ORs, GRs, and IRs—by a BLASTX search of the contigs against
319 amino acid sequences and Pfam domains of chemosensory receptors in *D.*
320 *melanogaster* (Table 2).

A homology search based on the Pfam domains of the 7tm odorant receptor (PF02949) and the amino acid sequences of the *Drosophila* ORs revealed 60 candidate ORs. The full-length coding sequences of 13 ORs were expected by *de novo* assembly. We also used a BLASTN search to predict the full-length coding sequences of three ORs—*BdorOR13a*, *BdorOR63a-2-v1*, and *BdorOR67c-v1*—and determined their sequences by RT-PCR. Interestingly, we found multiple homologous genes for several ORs including *BdorOR7a* and *BdorOR67d* (Table 2), whereas the corresponding *Drosophila* ORs have only one gene. The divergence of the *BdorOR7a* subfamily in the phylogenetic tree is remarkable (Fig. 1).

We identified four GRs that are homologous to sugar receptors such as the *GR5a* and *GR64* subfamilies by a homology search based on the Pfam domains of trehalose receptors (PF06151) (Freeman and Dahanukar, 2015). We identified another 13 GRs using the Pfam domains of 7tm chemosensory receptors (PF08395). We identified two GRs as *BdorGR21a* and *BdorGR63a*, which are carbon dioxide receptors and are highly conserved in insect species (Jones et al., 2007; Kwon et al., 2007). We also found four homologous genes of *BdorGR21a*. Apart from the trehalose and carbon dioxide receptors, we identified eight GRs from the Trinity contigs, one of which was a homolog of the fructose receptor *GR43a* (Sato et al., 2011).

We identified ligand-gated ion channels by a homology search based on the Pfam domains of ligand-gated ion channels (PF00060) and *Drosophila* IRs. Of those, we identified the candidate IRs by a BLASTP search based on translated protein sequences. Among the IRs, we identified two—*BdorIR8a* and *BdorIR25a*—as ionotropic co-receptors (Benton et al., 2009; Abuin et al., 2011). We also found a

345 homologous gene of *BdorIR8a*, and a pair of homologous genes in *BdorIR31a*,
346 *BdorIR64a*, *BdorIR75a*, *BdorIR76a*, *BdorIR92a*, and *BdorIR93a*.
347 Previous studies have identified candidate chemosensory receptors in *B.*
348 *dorsalis* (Wu et al., 2015; Liu et al., 2016). A comparison of those receptors with the
349 chemosensory receptors found in the present study indicated the novel ORs, GRs, and
350 IRs listed in Table 2. The coding sequences of the candidate chemosensory receptor
351 genes and their accession numbers are shown in the S2 text and Table S4,
352 respectively.
353
354 ***3.2. Expression profiles of the ORs***
355 Sex-specific behaviors—including attraction and oviposition responses to
356 plant semiochemicals—have been observed in *B. dorsalis* (Howlett, 1912; Nishida et
357 al., 1988; Siderhurst and Jang, 2006a; Siderhurst and Jang, 2006b; Kamala Jayanthi et
358 al., 2012; Kamala Jayanthi et al., 2014; Damodaram et al., 2014). Therefore, we used
359 qPCR to compare the transcription levels of 15 ORs with known full-length coding
360 sequences in female and male antennae to determine if expression was sex-specific.
361 All the ORs tested were expressed in both sexes, although we did observe a
362 significant difference in the transcription level of *BdorOR35a* between the female and
363 male antennae (Fig. 2).
364 The expression of candidate chemosensory receptors in the male antenna and
365 proboscises was predicted by FPKM analysis (Table S5). Whereas most of the
366 transcripts coding ORs had various FPKM values in the antennae, some including
367 *BdorORCO* also had relatively low FPKM values in the proboscises. With regard to
368 the GRs, the transcripts coding carbon dioxide receptors—namely *BdorGR21a* and its
369 variants—had high FPKM values in the antennae. Some of the transcripts coding

GRs—including the sugar receptor subfamilies *BdorGR5a* and *BdorGR64*—only had high FPKM values in the proboscises, whereas the transcripts coding *BdorGR28b* and *BdorGR8a* had FPKM values in the antennae, but not in the proboscises. Most of the transcripts coding IRs had various FPKM values in the antennae, and some had FPKM values in the proboscises. In contrast, the transcripts coding *BdorIR56c* and *BdorIR93a-2* only had FPKM values in the proboscises.

We used RT-PCR to examine the transcription profile of *BdorORCO* in various tissues including olfactory and gustatory organs (Fig. 3A). *BdorORCO* was expressed primarily in the olfactory organs, antennae, and maxillary palps, although we observed marginal expression in the proboscises. We used qPCR to quantitatively compare the transcription levels of this gene in female and male antennae, proboscises, and tarsi (Fig. 3B). *BdorORCO* was highly expressed in both female and male antennae in accordance with its role as an obligatory co-receptor. In contrast, the transcription levels of *BdorORCO* were extremely low in both female and male proboscises (less than one thousandth of those in the antennae). The transcription levels of *BdorORCO* in both female and male tarsi were as low as one tenth those in the proboscises.

We also used RT-PCR to analyze the expression profiles of the selected ORs *BdorOR13a* and *BdorOR82a* (Fig. 3A). The PCR products of these genes were detected by 40 cycles of amplification, but not by 35 cycles, probably owing to the low transcription levels. We observed *BdorOR13a* expression in both female and male antennae, and in male maxillary palps and gustatory organs, i.e., the proboscises and foreleg tarsi. We observed *BdorOR82a* expression in both female and male antennae, and in male maxillary palps and female foreleg tarsi. We used qPCR to compare transcription levels in females and males to determine if *BdorOR13a* and

BdorOR82a were expressed in a sexually dimorphic pattern. There were extremely low *BdorOR13a* and *BdorOR82a* transcription levels in both the proboscises and foreleg tarsi (Fig. 3C). There were no significant differences in the transcription levels of these genes between female and male proboscises or foreleg tarsi ($p > 0.05$, Student's *t*-test).

3.3. Identification of ligands for BdorOR13a and BdorOR82a by two-electrode voltage-clamp recording

To identify the ligands for the ORs in *B. dorsalis*, we co-expressed each of the ten receptor proteins—BdorOR7a-4, BdorOR7a-7, BdorOR13a, BdorOR35a, BdorOR43a-2-v1, BdorOR63a-2-v1, BdorOR67c-v1, BdorOR67d-1, BdorOR74a and BdorOR82a— with the obligatory co-receptor BdorORCO in *Xenopus* oocytes. We tested 24 compounds including host plant chemicals, male attractants, and sex pheromones for *Bactrocera* species, as shown in Table S3 and Fig. S1. Of the ten receptor proteins tested, BdorOR13a responded to one compound and BdorOR82a responded to three compounds. The oocyte co-expressing BdorOR13a with BdorORCO responded significantly to 1-octen-3-ol at a concentration of 100 μM (Fig. 4A-C). The current value induced by 1-octen-3-ol was significantly higher than that induced by the control (DMSO). The current induced by 1-octen-3-ol increased in a dose-dependent manner, and the EC_{50} value was 52.0 μM (Fig. 4D, E). The oocyte co-expressing BdorOR82a and BdorORCO responded significantly to geranyl acetate, and responded weakly to farnesenes and linalyl acetate at a concentration of 100 μM (Fig. 5A-C). Further experiments revealed significant differences between the current values of these compounds and that of the control (Fig. 5D). The current induced by

geranyl acetate increased in accordance with an increase in concentration, but we did not observe a plateau at the maximum concentration tested (Fig. 5E, F).

We compared the amino acid sequences of the characterized ORs—BdorOR13a and BdorOR82a—with those of the *Drosophila* ORs because the properties of these ORs have been well characterized in *D. melanogaster*. BLASTP analysis indicated that the deduced amino acid sequences of BdorOR13a and BdorOR82a were similar to the sequences of DmOR13a (GenBank: AAF48549.2) and DmOR82a (GenBank: AAN13335.1), with 51% and 43% amino acid identities, respectively. Alignments of these ORs revealed that the amino acids within the transmembrane domains are well conserved (Fig. S3).

3.4 Behavioral bioassay

We examined the effect of volatiles characterized as ligands for BdorOR13a and BdorOR82a on the landing behavior of *B. dorsalis*. To determine whether there were behavioral differences between the sexes in terms of mating, we tested both females and males individually with four volatiles—1-octen-3-ol, geranyl acetate, farnesenes, and linalyl acetate—before and after mating. Because hardly any flies were attracted to the volatile-treated filter papers when used alone, we placed two sets of green and white spheres in the cage as visual cues, each containing microtubes with filter papers treated with or without the aforementioned volatiles (Fig. S2). We attempted to determine whether volatiles or color affected the number of flies landing on each sphere using the GLM model. When the fruit flies were exposed to 1-octen-3-ol—the ligand for BdorOR13a—the volatile factor but not the color factor alone significantly affected the numbers of mated females, and the numbers of both virgin and mated males landing on the spheres (Table 3). The number of mated females landing on the

spheres increased by the exposure to 1-octen-3-ol (Fig. 6A). Furthermore, we observed a few of the mated females probing the surface of the sphere with abdominal bending and aculeus extension, which are typical oviposition behaviors. However, the numbers of both virgin and mated males decreased by the exposure to 1-octen-3-ol (Fig. 6A). When the fruit flies were exposed to geranyl acetate or farnesenes—the ligands for BdorOR82a—the volatile factor but not the color factor alone significantly affected the behavior of the mated females, and more of them landed on the spheres emitting the volatiles (Table 3, Fig. 6A). In contrast, the number of mated males landing on the spheres decreased by the exposure to farnesenes. When the fruit flies were exposed to linalyl acetate, the volatile factor did not affect the number of fruit flies landing on the spheres, but color alone significantly increased the number of mated females (Table 3). Taken together, these results indicate that exposure to each of the volatiles—namely 1-octen-3-ol, geranyl acetate and farnesenes—significantly affected the landing behavior of the mated *B. dorsalis* females, whereas the males seemed to avoid the spheres that emitted 1-octen-3-ol or farnesenes. We confirmed that exposure to ethanol as a solvent did not affect the landing behavior of the mated females (Fig. S4A). Although the total number of mated females landing on the four spheres emitting 1-octen-3-ol, geranyl acetate, or farnesenes was significantly higher than in the other groups (Fig. 6B), there was no significant difference in the number of females landing on the spheres when the volatile-emitting and ethanol-emitting spheres (the control) were compared (Fig. S4B). This suggests that mated females frequently landed on spheres regardless of whether they were emitting a volatile.

4. Discussion

4.1. Repertoires of chemosensory receptor families in B. dorsalis

We identified multiple candidate chemosensory receptors in *B. dorsalis*—including novel ORs, GRs, and IRs not reported in previous studies—by transcriptome analysis. We found divergent homologs and variants in several ORs from *B. dorsalis*, suggesting that ancient genes have diverged by gene duplication in these OR families during adaptation to environmental odorants such as plant volatiles. The physiological roles of the highly divergent BdorOR7a family are of particular interest because *B. dorsalis* seems to require homologous ORs to detect specific odorants or sets of similar odorants. With regard to GRs, we identified two highly conserved carbon dioxide receptors, sugar receptors, and several other receptors. We identified 23 IRs including the homologs of *Drosophila* ionotropic co-receptors IR8a and IR25a. We found several IRs with two variants—e.g., BdorIR75a, BdorIR76a and BdorIR93a—suggesting that gene duplication has occurred in these IR families, as in the ORs. It is interesting to note that *Drosophila* OR67d and GR32a have been characterized as receptors for volatile and contact pheromones, respectively (Kohl et al., 2015). OR67d functions as a receptor for 11-*cis*-vaccenyl acetate (Ha, 2006; Kurtovic et al., 2007); this compound acts as an anti-aphrodisiac pheromone in males to avoid male–male courtship (Zawistowski and Richmond, 1986), but also acts as an aggregation signal in both sexes (Bartelt et al., 1985). The aggregation behavior of males—known as lek formation—has been observed in *B. dorsalis* and related species (Iwahashi and Majima, 1986; Tan and Nishida, 1996). Therefore, it would be intriguing if the divergent receptors (i.e., members of the BdorOR67d family) were involved in such social behavior.

4.2. Expression profiles of chemosensory receptors of B. dorsalis

493 In *Drosophila*, OR genes are expressed exclusively in olfactory organs (Vosshall et
494 al., 2000), and GR genes are mainly expressed in gustatory organs, with some
495 exceptions such as the expression of *GR21a*, *GR63a*, and *GR22e* in antennae (Scott et
496 al., 2001; Dunipace et al., 2001; Thorne and Amrein, 2008). In contrast, we acquired
497 sequences coding multiple ORs from reads derived from the proboscises, although the
498 FPKM values of these transcripts were low. Likewise, we found transcripts of several
499 GR genes in the sequencing reads from the antennae. Among these, *BdorGR8a* and
500 *BdorGR28b* had relatively high FPKM values in the antennae, but no FPKM values in
501 the proboscises. Therefore, differences in the expression profiles of several OR and
502 GR genes in the olfactory and gustatory organs are more obscure in *B. dorsalis* than in
503 *D. melanogaster*. Because the males of many *Bactrocera* species are strongly
504 attracted to specific compounds—e.g., *B. dorsalis* to ME—and subsequently feed
505 voraciously on the compounds, the perception of attractants probably involves both
506 olfactory and gustatory stimulation. A previous study demonstrated that ORCO is
507 required for the attraction of *B. dorsalis* to ME (Zheng et al., 2012), suggesting the
508 involvement of ORs in ME reception. It is also possible that IRs mediate the detection
509 of male attractants in *B. dorsalis*, because IRs function as both olfactory and gustatory
510 receptors (Rytz et al., 2013; Fleischer et al., 2017). In the present study, although we
511 found most transcripts of *BdorIRs* in sequencing reads from the antennae, we found
512 others in reads from both antennae and proboscises, or from proboscises only. The
513 question of whether ORs, GRs, IRs, or some combination of them is required for the
514 chemoreception of the attractants is very interesting.
515 Although sexually dimorphic behavior in response to semiochemicals has been
516 reported in *B. dorsalis* (Howlett, 1912; Nishida et al., 1988; Tan and Nishida, 2012),
517 in the present study we found no distinction between females and males in terms of

518 the transcription levels of almost all the ORs tested. Conversely, in lepidopteran
519 species there is sex-specific expression of the chemosensory receptors required for
520 sexually dimorphic behavior such as mating or oviposition in chemosensory organs.
521 Since the characterization of a sex pheromone receptor from the silkworm *Bombyx*
522 *mori* (Sakurai et al., 2004; Nakagawa et al., 2005), lepidopteran receptors that
523 perceive female pheromones, all of which are specifically expressed in male antennae,
524 have been identified in various genera including *Plutella*, *Mythimna*, *Diaphania*,
525 *Antheraea*, and *Ostrinia* (Mitsuno et al., 2008; Forstner et al., 2009; Miura et al.,
526 2009; Miura et al., 2010; Wanner et al., 2010). Other than sex pheromone receptors,
527 the female-specific expression of lepidopteran gustatory receptor for the detection of
528 an oviposition stimulant contained in host plant leaves has been reported in the
529 swallowtail butterfly *Papilio xuthus* (Ozaki et al., 2011). These findings suggest that
530 the sex-specific expression of chemosensory receptors is closely related to sexually
531 dimorphic behavior in lepidopteran species. In *D. melanogaster*, however,
532 chemosensory receptor genes are mostly expressed in both sexes, whereas the
533 gustatory pheromone receptor GR68a is specifically expressed in male taste neurons
534 in the foreleg tarsi (Bray and Amrein, 2003). Therefore, the chemosensory
535 information involved in sexually dimorphic behavior triggered by semiochemicals is
536 processed by the central nervous system, rather than the peripheral, in dipteran species
537 including *B. dorsalis*.
538
539 ***4.3. Binding properties of ORs that respond to plant volatiles***
540 In the present study, we characterized two ORs that respond to plant volatiles using
541 *Xenopus* oocytes as a heterologous expression system. BdorOR13a responded
542 strongly to 1-octen-3-ol, as reported in its homologs in *D. melanogaster* and the

mosquito species *Anopheles gambiae* and *Aedes aegypti* (Lu et al., 2007; Kreher et al., 2008; Bohbot and Dickens, 2009). BdorOR82a responded to geranyl acetate, as reported in its homologs in *D. melanogaster* and *A. gambiae* (Hallem et al., 2004; Wang et al., 2010). BdorOR82a also responded significantly to farnesenes and linalyl acetate, whereas a response to these compounds by the homologous OR in *D. melanogaster* has not been reported, to the best of our knowledge. Whereas the response of BdorOR13a to 1-octen-3-ol reached a plateau at an approximate concentration of 100 μM, the response of BdorOR82a to geranyl acetate failed to reach a plateau up to 10 mM, the maximum concentration tested. Furthermore, BdorOR82a responded weakly to farnesenes and linalyl acetate at 100 μM. Whereas 1-octen-3-ol contains a hydroxyl group, geranyl acetate, the isomers of farnesenes and linalyl acetate lack hydrophilic groups, suggesting that the latter three compounds are relatively insoluble in buffer solutions owing to their hydrophobicity. The sparing solubility of geranyl acetate in buffer solution may explain the weak response of BdorOR82a, even at 1 mM. Generally, odorant-binding proteins allow hydrophobic ligands to access the receptor neurons of insect chemosensilla, which are surrounded by an aqueous lymphatic fluid (Fleischer et al., 2017). Therefore, geranyl acetate, farnesenes, and linalyl acetate could effectively access BdorOR82a on receptor neurons mediated by binding proteins *in vivo*. An alternative explanation for the weak response is the low affinity of BdorOR82a for geranyl acetate. Because the amino acid identity between BdorOR82a and DmOR82a is not necessarily high, differences in amino acid residues may affect the sensitivity and specificity of the heteromeric insect OR82a/ORCO complex.

BdorOR82a responded to the three hydrophobic compounds. Geranyl acetate and linalyl acetate are both isomeric monoterpenes, but linalyl acetate is branched in a

568 different way. Farnesenes are sesquiterpene hydrocarbons that differ from geranyl
569 acetate and linalyl acetate in both size and nature. Of the compounds that have been
570 tested, the *D. melanogaster* DmOR82a homolog responds exclusively to geranyl
571 acetate (Hallem et al., 2004; Hallem and Carlson, 2006), but it is unclear whether
572 DmOR82a also responds to linalyl acetate and farnesenes. Although BdorOR82a has
573 a low E-value (4e-98) against DmOR82a according to a BLASTP search, the amino
574 acid sequences of BdorOR82a and DmOR82a have only 43% identity. Therefore, it
575 would be interesting if DmOR82a responds to linalyl acetate and farnesenes as does
576 BdorOR82a. A comparison of the binding affinities and amino acid sequences of
577 BdorOR82a and DmOR82a would provide information about the specificities of these
578 receptors.
579
580 ***4.4. Biological function of volatiles characterized as ligands for BdorORs***
581 We found that three ligands for BdorORs—namely, 1-octen-3-ol, geranyl acetate and
582 farnesenes—significantly affect the landing behavior of adult flies. The results
583 suggest that adult flies respond to plant volatiles via the ORs characterized in the
584 present study. However, it should be noted that at least one other OR may be involved
585 in information processing at the peripheral and/or central nervous system level during
586 the response to these volatiles.
587 Interestingly, the ligands had different effects depending on the sex and
588 mating status of the flies. For example, 1-octen-3-ol increased the number of mated
589 females landing on the spheres but reduced the numbers of virgin and mated males
590 landing on the spheres. It should be noted that 1-octen-3-ol has been identified as an
591 oviposition stimulant of *B. dorsalis* in studies on the volatile components of a host
592 fruit (mango; *Mangifera indica*); gravid females laid more eggs on discs treated with

1-octen-3-ol in binary choice tests (Kamala Jayanthi et al., 2014). We also found that a few of the mated females exhibited oviposition behavior on the surfaces of the spheres emitting 1-octen-3-ol. Our findings suggest that in tephritids, mating causes a switch from normal to oviposition-related behavior, as observed in *B. dorsalis*. A study on female Mediterranean fruit flies (*Ceratitis capitata*) has also demonstrated that mating confers a preferential switch; mated females choose host fruit odor over male pheromones (Jang, 1995). The negative effects on the landing behavior of males, regardless of mating experience, suggest that information processing after the perception of volatiles differs between the sexes. It is worth noting that various dipteran behavioral responses to 1-octen-3-ol have been reported, e.g., its ability to attract *D. melanogaster* larvae (Kreher et al., 2008), and its ability to repel adult females of the spot wing drosophila *D. suzukii* (Wallingford et al., 2016). *Anopheles gambiae* and *Aedes aegypti* mosquitoes are attracted to 1-octen-3-ol in the breath of animals (Kline, 1994). It would be interesting to know if OR13a homologs are associated with these different behaviors in dipteran species, or if another receptor is involved.

Geranyl acetate, farnesenes, and linalyl acetate, which are ligands for BdorOR82a, have been detected in the tropical almond fruit *Terminalia catappa*, one of the hosts of *B. dorsalis* (Siderhurst and Jang, 2006b); geranyl acetate and methyl eugenol elicited the largest electroantennogram detection (EAD) responses from the antennae of *B. dorsalis* among the volatiles collected from *T. catappa* (Siderhurst and Jang, 2006b). Linalyl acetate and the isomers of farnesenes also elicited EAD responses to some extent (Siderhurst and Jang, 2006b). In accordance with the EAD responses, exposure to geranyl acetate or farnesenes had a significant effect on the landing behavior of *B. dorsalis* in our experiments, and we observed different

618 responses to 1-octen-3-ol with regard to gender and mating experience. These results
619 suggest that the electrophysiological responses to geranyl acetate and farnesenes are
620 linked to their effects on the landing behavior of fruit flies.

621 Geranyl acetate also seems to be important for *D. melanogaster*, because
622 DmOR82a responds to its analog geranyl acetone, probably as a signal for fruit
623 ripening (Mansourian and Stensmyr, 2015). It would be interesting to see if OR82a
624 homologs, which respond to geranyl acetate and its analogs, commonly mediate
625 semiochemical information released from host fruits in fruit flies of the families
626 Drosophilidae and Tephritidae. Recently, ORs that respond to geranyl acetate and an
627 isomer of farnesene have been identified in aphid and moth species (Liu et al., 2014;
628 Zhang et al., 2017), although these receptors do not belong to the OR82a family.
629 ApisOR5 from the aphid *Acyrthosiphon pisum* responds to the alarm pheromone (*E*)-
630 β-farnesene and the repellent geranyl acetate, even though there is no homology
631 between ApisOR5 and OR82a at the amino acid level. Instead, ApisOR5 most closely
632 resembles OR85f, with an E-value of 1.6e-4 according to a BLASTP search against
633 *Drosophila* ORs. Similarly, SexiOR3, which has been identified in the beet
634 armyworm moth *Spodoptera exigua*, responds to (*E*)-β-farnesene, but most closely
635 resembles OR13a, with an E-value of 7.9e-6 according to a BLASTP search against
636 *Drosophila* ORs. The relationships between the binding properties and structures of
637 these ORs, which share common ligands, are intriguing.
638 To the best of our knowledge, the present study was the first attempt to functionally
639 characterize the ORs of tephritid fruit flies using *Xenopus* oocytes as a heterologous
640 expression system. The results will enable us to further characterize the orphan
641 receptors of Tephritidae. Furthermore, the identification of ligands for chemosensory
642 receptors will provide information about the important chemicals that affect the life

643 cycles of fruit flies. Despite the characterization of the BdorORs, we have not
644 provided direct evidence to link the properties of ORs with behavior as an output of
645 signal processing mediated by these receptors. Insect genome engineering using the
646 CRISPR/Cas9 system is now available for *Bactrocera* species (Choo et al., 2017), and
647 the loss of the function of specific chemosensory receptors of interests will clarify
648 their roles *in vivo*. This will lead to the elucidation of the mechanisms underlying the
649 chemoreception of various semiochemicals, including plant volatiles, male-specific
650 attractants, and sex pheromones.
651

652 ## Acknowledgements

653 We thank Isao Miyazaki, Hiroyuki Adachi and Takuji Yamamoto of Naha Plant
654 Protection Station (Okinawa, Japan) for providing the fruit flies. We also thank
655 Shinya Kanno, Yurie Hirosaki, Farhana Ainie, and Nor Nasuha for technical
656 assistance. HO was supported by JSPS KAKENHI Grant Number 26450466.

Figure captions

Fig. 1. Phylogenetic tree of candidate olfactory receptors (ORs) identified in *Bactrocera dorsalis*. Branch length is proportional to genetic distance estimated by the maximum likelihood method. The values shown at the nodes of the branches are bootstrap values (%) from 1000 replicate samplings. The numbers in parentheses indicate the numbers of amino acids in the ORs. “F” or “P” in parentheses indicate full or partial determination of the coding sequence of an OR, respectively. ORs expressed in *Xenopus* oocytes are indicated in bold.

Fig. 2. Transcription levels of olfactory receptors (ORs) identified in *Bactrocera dorsalis*. FA: female antennae; MA: male antennae. Each value is plotted as a dot (n = 6). The box plot shows 25–75% (box), median (band inside), and minima to maxima (whiskers). Student’s t-test: $*p < 0.05$.

Fig. 3. Expression patterns of olfactory receptors (ORs) identified in *Bactrocera dorsalis*. (A) Expression of ORs in various tissues detected by RT-PCR. PCR amplifications were performed in 35 cycles for *BdorORCO* and *rpS3*, and 40 cycles for *BdorOR13a* and *BdorOR82a*. The lanes are as follows: AT: antennae; MP: maxillary palps; PB: proboscises; FT: foreleg tarsi; ML: midlegs; HL: hindlegs. (B) Transcription levels of *BdorORCO* in chemosensory organs. (C) Transcription levels of *BdorOR13a* and *BdorOR82a* in gustatory organs. (B, C) FA: female antennae; MA: male antennae; FP: female proboscises; MP: male proboscises; FT: female tarsi; MT: male tarsi. Each value is plotted as a dot (n = 5–6). The box plot shows 25–75% (box), median (band inside), and minima to maxima (whiskers).

683

684 Fig. 4. Responses of *Xenopus* oocytes expressing BdorOR13a with BdorORCO to
685 various compounds. (A) Current trace of an oocyte upon successive exposures to 25
686 samples including DMSO (the control). Each chemical was applied at the time
687 indicated by the arrowhead. (B) Currents measured in the oocytes. The structure of
688 each compound and its corresponding abbreviation is shown in Fig. S1. The number
689 in parentheses after each compound corresponds to the number on the arrowhead in
690 (A). Error bars indicate SE (n = 3). Student's t-test: *$p < 0.05$. (C) Structure of a
691 ligand for BdorOR13a. (D) Responses of an oocyte expressing BdorOR13a with
692 BdorORCO to 1-octen-3-ol at various concentrations. (E) Dose–response curve of
693 oocytes responding to 1-octen-3-ol. Each point represents the mean current value.
694 Error bars indicate SE (n = 9–11).

695

696 Fig. 5. Responses of *Xenopus* oocytes expressing BdorOR82a with BdorORCO to
697 various compounds. (A) Current trace of an oocyte upon successive exposures to 25
698 samples including DMSO (the control). Each chemical was applied at the time
699 indicated by the arrowhead. (B) Currents measured in the oocytes. The structure of
700 each compound and its corresponding abbreviation is shown in Fig. S1. The number
701 in parentheses after each compound corresponds to those on the arrowheads in (A).
702 Error bars indicate SE (n = 3). Student's t-test: *$p < 0.05$. (C) Structures of ligands for
703 BdorOR82a. The structure of one of the farnesene isomers is shown. (D) Currents
704 measured in oocytes responding to L-OAc (linalyl acetate), FRN-mix (a mixture of
705 farnesene isomers), and G-OAc (geranyl acetate) at 100 μM. Each value is plotted as
706 a dot (n = 9–10). The box plot shows 25–75% (box), median (band inside), and
707 minima to maxima (whiskers). Student's t-test: *$p < 0.05$, **$p < 0.01$. (E) Responses

of an oocyte expressing BdorOR82a with BdorORCO to geranyl acetate at various concentrations. (F) Dose–response curve of oocytes responding to geranyl acetate. Each point represents the mean current value. Error bars indicate SE (n = 8–9).

Fig. 6. Effects of volatiles and visual cues on landing behaviors of *Bactrocera dorsalis*. The box plot shows 25–75% (box), median (band inside), and minima to maxima (whiskers). Virgin-F, Mated-F, Virgin-M, and Mated-M indicate virgin females, mated females, virgin males, and mated males, respectively. (A) Numbers of virgin or mated females or males landing on green or white spheres. The numbers of flies are plotted as dots (n = 5–6). Significant effects of volatiles or colors indicated in Table 3 are shown in the categories of fruit flies as V or C with p-values (< 0.05), respectively. T-G, T-W, C-G, and C-W indicate volatile-treated green balls, volatile-treated white balls, control (volatile-untreated) green balls, and control white balls, respectively. (B) Total numbers of fruit flies landing on the spheres calculated from (A). Boxes with letters are significantly different at $p < 0.05$ according to Tukey's HSD test.

References

Abuin, L., Bargeton, B., Ulbrich, M.H., Isacoff, E.Y., Kellenberger, S., Benton, R., 2011. Functional architecture of olfactory ionotropic glutamate receptors. Neuron 69, 44–60. doi:10.1016/j.neuron.2010.11.042

Bartelt, R.J., Schaner, A.M., Jackson, L.L., 1985. *cis*-Vaccenyl acetate as an aggregation pheromone in *Drosophila melanogaster*. J. Chem. Ecol. 11, 1747–1756. doi:10.1007/BF01012124

Benton, R., Vannice, K.S., Gomez-Diaz, C., Vosshall, L.B., 2009. Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. Cell 136, 149–162. doi:10.1016/j.cell.2008.12.001

Bohbot, J.D., Dickens, J.C., 2009. Characterization of an enantioselective odorant receptor in the yellow fever mosquito *Aedes aegypti*. PLoS One 4(9), e7032. doi:10.1371/journal.pone.0007032

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic : a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. doi:10.1093/bioinformatics/btu170

Bray, S., Amrein, H., 2003. A putative *Drosophila* pheromone receptor expressed in male-specific taste neurons is required for efficient courtship. Neuron 39, 1019–1029. doi:10.1016/S0896-6273(03)00542-7

Choo, A., Crisp, P., Saint, R., O'Keefe, L.V., Baxter, S.W., 2017. CRISPR/Cas9-mediated mutagenesis of the *white* gene in the tephritid pest *Bactrocera tryoni*. J. Appl. Entomol. 142, 1–7. doi:10.1111/jen.12411

Damodaram, K.J.P., Kempraj, V., Aurade, R.M., Venkataramanappa, R.K., Nandagopal, B., Verghese, A., Bruce, T., 2014. Oviposition site-selection by *Bactrocera dorsalis* is mediated through an innate recognition template tuned to γ-octalactone. PLoS One 9(1), e85764. doi:10.1371/journal.pone.0085764

Dunipace, L., Meister, S., McNealy, C., Amrein, H., 2001. Spatially restricted expression of candidate taste receptors in the *Drosophila* gustatory system. Curr. Biol. 11, 822–835. doi:10.1016/S0960-9822(01)00258-5

Enomoto, H., Ishida, T., Hamagami, A., Nishida, R., 2010. 3-Oxygenated α-ionone derivatives as potent male attractants for the solanaceous fruit fly,

758 *Bactrocera latifrons* (Diptera: Tephritidae), and sequestered metabolites in
759 the rectal gland. Appl. Entomol. Zool. 45, 551–556. doi:10.1303/aez.2010.551
760 Fleischer, J., Pregitzer, P., Breer, H., Krieger, J., 2017. Access to the odor world:
761 olfactory receptors and their role for signal transduction in insects. Cell.
762 Mol. Life Sci. 1–24. doi:10.1007/s00018-017-2627-5
763 Forstner, M., Breer, H., Krieger, J., 2009. A receptor and binding protein
764 interplay in the detection of a distinct pheromone component in the
765 silkmoth *Antheraea polyphemus*. Int. J. Biol. Sci. 5, 745–757.
766 Freeman, E.G., Dahanukar, A., 2015. Molecular neurobiology of *Drosophila*
767 taste. Curr. Opin. Neurobiol. 34, 140–148. doi:10.1016/j.conb.2015.06.001
768 Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I.,
769 Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E.,
770 Hacohen, N., Gnirke, A., Rhind, N., Di Palma, F., Birren, B.W., Nusbaum,
771 C., Lindblad-Toh, K., Friedman, N., Regev, A., 2011. Full-length
772 transcriptome assembly from RNA-Seq data without a reference genome.
773 Nat. Biotechnol. 29, 644–652. doi:10.1038/nbt.1883
774 Ha, T.S., Smith, D.P., 2006. A pheromone receptor mediates 11-*cis*-vaccenyl
775 acetate-induced responses in *Drosophila*. J. Neurosci. 26, 8727–8733.
776 doi:10.1523/JNEUROSCI.0876-06.2006
777 Hallem, E.A., Carlson, J.R., 2006. Coding of odors by a receptor repertoire. Cell
778 125, 143–160. doi:10.1016/j.cell.2006.01.050
779 Hallem, E.A, Ho, M.G., Carlson, J.R., 2004. The molecular basis of odor coding
780 in the *Drosophila* antenna. Cell 117, 965–79. doi:10.1016/j.cell.2004.05.012
781 Howlett, F., 1912. Chemical reactions of fruit-flies. Bull. Entomol. Res. 6, 297–
782 305. doi:10.1017/S0007485300000328
783 Iwahashi, O., Majima, T., 1986. Lek formation and male-male competition in the
784 melon fly, *Dacus cucurbitae* Coquillett (Diptera : Tephritidae). Appl.
785 Entomol. Zool. 21, 70–75. doi:http://doi.org/10.1303/aez.21.70
786 Jang, E.B., 1995. Effects of mating and accessory gland injections on olfactory-
787 mediated behavior in the female Mediterranean fruit fly, *Ceratitis capitata*.
788 J. Insect Physiol. 41, 705–710. doi:10.1016/0022-1910(95)00015-M
789 Jones, W.D., Cayirlioglu, P., Kadow, I.G., Vosshall, L.B., 2007. Two
790 chemosensory receptors together mediate carbon dioxide detection in
791 *Drosophila*. Nature 445, 86–90. doi:10.1038/nature05466

792 Kamala Jayanthi, P.D., Kempraj, V., Aurade, R.M., Venkataramanappa, R.K.,
793 Nandagopal, B., Verghese, A., Bruce, T.J.A., 2014. Specific volatile
794 compounds from mango elicit oviposition in gravid *Bactrocera dorsalis*
795 females. J. Chem. Ecol. 40, 259–266. doi:10.1007/s10886-014-0403-7
796 Kamala Jayanthi, P.D., Woodcock, C.M., Caulfield, J., Birkett, M.A., Bruce,
797 T.J.A., 2012. Isolation and identification of host cues from mango,
798 *Mangifera indica*, that attract gravid female oriental fruit fly, *Bactrocera*
799 *dorsalis*. J. Chem. Ecol. 38, 361–369. doi:10.1007/s10886-012-0093-y
800 Kline, D.L., 1994. Olfactory attractants for mosquito surveillance and control: 1-
801 Octen-3-ol. J. Am. Mosq. Control Assoc. 10, 280–287.
802 Kohl, J., Huoviala, P., Jefferis, G.S.X.E., 2015. Pheromone processing in
803 *Drosophila*. Curr. Opin. Neurobiol. 34, 149–157.
804 doi:10.1016/j.conb.2015.06.009
805 Kreher, S.A., Mathew, D., Kim, J., Carlson, J.R., 2008. Translation of sensory
806 input into behavioral output via an olfactory system. Neuron 59, 110–124.
807 doi:10.1016/j.neuron.2008.06.010
808 Kurtovic, A., Widmer, A., Dickson, B.J., 2007. A single class of olfactory neurons
809 mediates behavioural responses to a *Drosophila* sex pheromone. Nature 446,
810 542–546. doi:10.1038/nature05672
811 Kwon, J.Y., Dahanukar, A., Weiss, L.A., Carlson, J.R., 2007. The molecular
812 basis of CO2 reception in *Drosophila*. Proc. Natl. Acad. Sci. U.S.A. 104,
813 3574–3578. doi:10.1073/pnas.0700079104
814 Liu, C., Liu, Y., Guo, M., Cao, D., Dong, S., Wang, G., 2014. Narrow tuning of an
815 odorant receptor to plant volatiles in *Spodoptera exigua* (Hübner). Insect
816 Mol. Biol. 23, 487–496. doi:10.1111/imb.12096
817 Liu, Z., Smagghe, G., Lei, Z., Wang, J.J., 2016. Identification of male- and
818 female-specific olfaction genes in antennae of the oriental fruit fly
819 (*Bactrocera dorsalis*). PLoS One 11(2): e0147783.
820 doi:10.1371/journal.pone.0147783
821 Lu, T., Qiu, Y.T., Wang, G., Kwon, J.Y., Rutzler, M., Kwon, H.W., Pitts, R.J.,
822 van Loon, J.J.A., Takken, W., Carlson, J.R., Zwiebel, L.J., 2007. Odor
823 coding in the maxillary palp of the malaria vector mosquito *Anopheles*
824 *gambiae*. Curr. Biol. 17, 1533–1544. doi:10.1016/j.cub.2007.07.062

825 Mansourian, S., Stensmyr, M.C., 2015. The chemical ecology of the fly. Curr.
826 Opin. Neurobiol. 34, 95–102. doi:10.1016/j.conb.2015.02.006
827 Metcalf, R., 1990. Chemical ecology of Dacinae fruit flies (Diptera: Tephritidae).
828 Ann. Entomol. Soc. Am. 83, 1017–1030.
829 Mitsuno, H., Sakurai, T., Murai, M., Yasuda, T., Kugimiya, S., Ozawa, R.,
830 Toyohara, H., Takabayashi, J., Miyoshi, H., Nishioka, T., 2008.
831 Identification of receptors of main sex-pheromone components of three
832 Lepidopteran species. Eur. J. Neurosci. 28, 893–902. doi:10.1111/j.1460-
833 9568.2008.06429.x
834 Miura, N., Nakagawa, T., Tatsuki, S., Touhara, K., Ishikawa, Y., 2009. A male-
835 specific odorant receptor conserved through the evolution of sex
836 pheromones in *Ostrinia* moth species. Int. J. Biol. Sci. 5, 319–330.
837 doi:10.7150/ijbs.5.319
838 Miura, N., Nakagawa, T., Touhara, K., Ishikawa, Y., 2010. Broadly and
839 narrowly tuned odorant receptors are involved in female sex pheromone
840 reception in *Ostrinia* moths. Insect Biochem. Mol. Biol. 40, 64–73.
841 doi:10.1016/j.ibmb.2009.12.011
842 Nakagawa, T., Sakurai, T., Nishioka, T., Touhara, K., 2005. Insect sex-
843 pheromone signals mediated by specific combinations of olfactory receptors.
844 Science 307, 1638–1642. doi:10.1126/science.1106267
845 Nishida, R., 2002. Sequestration of defensive substances from plants by
846 Lepidoptera. Annu. Rev. Entomol. 47, 57–92.
847 doi:10.1146/annurev.ento.47.091201.145121
848 Nishida, R., Tan, K.H., Serit, M., Lajis, N.H., Sukari, A.M., Takahashi, S.,
849 Fukami, H., 1988. Accumulation of phenylpropanoids in the rectal glands of
850 males of the Oriental fruit fly, *Dacus dorsalis*. Experientia 44, 534–536.
851 Nishida, R., and Tan, K.H., 2016. Search for new fruit fly attractants from
852 plants: A review. Proceedings of the 9th International Symposium on Fruit
853 Flies of Economic Importance, 249-262. ISBN: 978-616-358-207-2.
854 Opitz, S.E.W., Müller, C., 2009. Plant chemistry and insect sequestration.
855 Chemoecology 19, 117–154. doi:10.1007/s00049-009-0018-6
856 Ozaki, K., Ryuda, M., Yamada, A., Utoguchi, A., Ishimoto, H., Calas, D.,
857 Marion-Poll, F., Tanimura, T., Yoshikawa, H., 2011. A gustatory receptor

involved in host plant recognition for oviposition of a swallowtail butterfly. Nat. Commun. 2, 542. doi:10.1038/ncomms1548

Reddy, G.V.P., Guerrero, A., 2004. Interactions of insect pheromones and plant semiochemicals. Trends Plant Sci. 9, 253–261. doi:10.1016/j.tplants.2004.03.009

Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2015. Dose-response analysis using R. PLoS One 10(12): e0146021, 1–13. doi:10.1371/journal.pone.0146021

Rytz, R., Croset, V., Benton, R., 2013. Ionotropic Receptors (IRs): Chemosensory ionotropic glutamate receptors in *Drosophila* and beyond. Insect Biochem. Mol. Biol. 43, 888–897. doi:10.1016/j.ibmb.2013.02.007

Sakurai, T., Nakagawa, T., Mitsuno, H., Mori, H., Endo, Y., Tanoue, S., Yasukochi, Y., Touhara, K., Nishioka, T., 2004. Identification and functional characterization of a sex pheromone receptor in the silkmoth *Bombyx mori*. Proc. Natl. Acad. Sci. U.S.A. 101, 16653–16658. doi:10.1073/pnas.0407596101

Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L.B., Touhara, K., 2008. Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature 452, 1002–1006. doi:10.1038/nature06850

Sato, K., Tanaka, K., Touhara, K., 2011. Sugar-regulated cation channel formed by an insect gustatory receptor. Proc. Natl. Acad. Sci. U.S.A. 108, 11680–11685. doi:10.1073/pnas.1019622108

Scott, K., Brady, R., Cravchik, A., Morozov, P., Rzhetsky, A., Zuker, C., Axel, R., 2001. A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila*. Cell 104, 661–673. doi:10.1016/S0092-8674(01)00263-X

Shelly, T., 2010. Effects of methyl eugenol and raspberry ketone/cue lure on the sexual behavior of *Bactrocera species* (Diptera : Tephritidae). Appl. Entomol. Zool. 45, 349–361. doi:10.1303/aez.2010.349

Siderhurst, M.S., Jang, E.B., 2006a. Attraction of female oriental fruit fly, *Bactrocera dorsalis*, to *Terminalia catappa* fruit extracts in wind tunnel and olfactometer tests. Formos. Entomol. 26, 45–55.

Siderhurst, M.S., Jang, E.B., 2006b. Female-biased attraction of oriental fruit fly, *Bactrocera dorsalis* (Hendel), to a blend of host fruit volatiles from

891 *Terminalia catappa* L. J. Chem. Ecol. 32, 2513–2524. doi:10.1007/s10886-
892 006-9160-6
893 Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011.
894 MEGA5: Molecular evolutionary genetics analysis using maximum
895 likelihood, evolutionary distance, and maximum parsimony methods
896 research resource. Mol. Biol. Evol. 28, 2731–2739.
897 doi:10.1093/molbev/msr121
898 Tan, K.H., Nishida, R., 1996. Sex pheromone and mating competition after
899 methyl eugenol consumption in the *Bactrocera dorsalis* complex. In:
900 McPheron, B.A., Steck, G.A., (eds) Fruit fly pests: a world assessment of
901 their biology and management. St. Lucie Press, Delray Beach, FL, pp 147–
902 153.
903 Tan, K.H., Nishida, R., 2012. Methyl eugenol : Its occurrence , distribution , and
904 role in nature, especially in relation to insect behavior and pollination. J.
905 Insect Sci. 12, 1–60.
906 Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the
907 sensitivity of progressive multiple sequence alignment through sequence
908 weighting, position-specific gap penalties and weight matrix choice. Nucleic
909 Acids Res. 22, 4673–4680.
910 Thorne, N., Amrein, H., 2008. Atypical expression of *Drosophila gustatory*
911 *receptor* genes in sensory and central neurons. J. Comp. Neurol. 506, 548–
912 568. doi:10.1002/cne
913 Touhara, K., Vosshall, L.B., 2009. Sensing odorants and pheromones with
914 chemosensory receptors. Annu. Rev. Physiol. 71, 307–332.
915 doi:10.1146/annurev.physiol.010908.163209
916 Vosshall, L.B., Stocker, R.F., 2007. Molecular architecture of smell and taste in
917 *Drosophila*. Annu. Rev. Neurosci. 30, 505–533.
918 doi:10.1146/annurev.neuro.30.051606.094306
919 Vosshall, L.B., Wong, A.M., Axel, R., 2000. An olfactory sensory map in the fly
920 brain. Cell 102, 147–159. doi:10.1016/S0092-8674(00)00021-0
921 Wallingford, A.K., Hesler, S.P., Cha, D.H., Loeb, G.M., 2016. Behavioral
922 response of spotted-wing drosophila, *Drosophila suzukii* Matsumura, to
923 aversive odors and a potential oviposition deterrent in the field. Pest Manag.
924 Sci. 72, 701–706. doi:10.1002/ps.4040

925 **Wang, G., Carey, A.F., Carlson, J.R., Zwiebel, L.J., 2010. Molecular basis of**
926 **odor coding in the malaria vector mosquito *Anopheles gambiae*. Proc. Natl.**
927 **Acad. Sci. 107, 4418–4423. doi:10.1073/pnas.0913392107**
928 **Wanner, K.W., Nichols, A.S., Allen, J.E., Bunger, P.L., Garczynski, S.F., Linn,**
929 **C.E., Robertson, H.M., Luetje, C.W., 2010. Sex pheromone receptor**
930 **specificity in the European corn borer moth, *Ostrinia nubilalis*. PLoS One**
931 **5(1): e8685. doi:10.1371/journal.pone.0008685**
932 **Wicher, D., Schäfer, R., Bauernfeind, R., Stensmyr, M.C., Heller, R.,**
933 **Heinemann, S.H., Hansson, B.S., 2008. *Drosophila* odorant receptors are**
934 **both ligand-gated and cyclic-nucleotide- activated cation channels. Nature**
935 **452, 1007–1011. doi:10.1038/nature06861**
936 **Wu, Z., Zhang, H., Wang, Z., Bin, S., He, H., Lin, J., 2015. Discovery of**
937 **chemosensory genes in the oriental fruit fly, *Bactrocera dorsalis*. PLoS One**
938 **10(6): e0129794. doi:10.1371/journal.pone.0129794**
939 **Yang, B., Ozaki, K., Ishikawa, Y., Matsuo, T., 2015. Identification of candidate**
940 **odorant receptors in asian corn borer *Ostrinia furnacalis*. PLoS One 10(3):**
941 **e0121261. doi:10.1371/journal.pone.0121261**
942 **Zawistowski, S., Richmond, R.C., 1986. Inhibition of courtship and mating of**
943 ***Drosophila melanogaster* by the male-produced lipid, *cis*-vaccenyl acetate. J.**
944 **Insect Physiol. 32, 189–192. doi:10.1016/0022-1910(86)90057-0**
945 **Zhang, R., Wang, B., Grossi, G., Falabella, P., Liu, Y., Yan, S., Lu, J., Xi, J.,**
946 **Wang, G., 2017. Molecular basis of alarm pheromone detection in aphids.**
947 **Curr. Biol. 27, 55–61. doi:10.1016/j.cub.2016.10.013**
948 **Zheng, W., Zhu, C., Peng, T., Zhang, H., 2012. *Odorant receptor co-receptor Orco***
949 **is upregulated by methyl eugenol in male *Bactrocera dorsalis* (Diptera:**
950 **Tephritidae). J. Insect Physiol. 58, 1122–1127.**
951 **doi:10.1016/j.jinsphys.2012.05.011**
952

Fig. 1

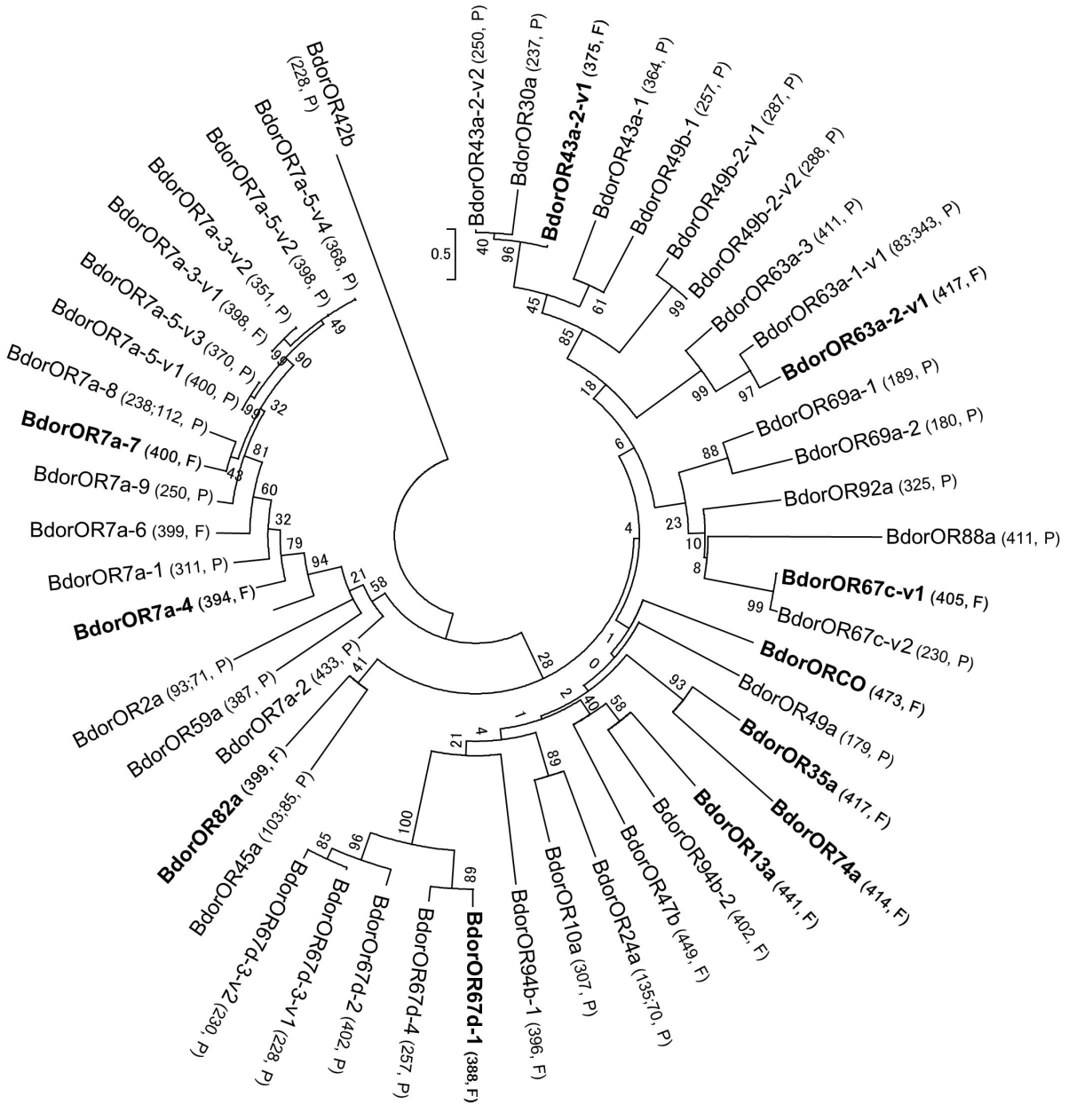

BdorOR42b (228, P)
BdorOR7a-5-v4 (368, P)
BdorOR7a-5-v2 (398, P)
BdorOR7a-3-v2 (351, P)
BdorOR7a-3-v1 (398, F)
BdorOR7a-5-v3 (370, P)
BdorOR7a-5-v1 (400, P)
BdorOR7a-8 (238;112, P)
BdorOR7a-7 (400, F)
BdorOR7a-9 (250, P)
BdorOR7a-6 (399, F)
BdorOR7a-1 (311, P)
BdorOR7a-4 (394, F)
BdorOR2a (93;71, P)
BdorOR59a (387, P)
BdorOR7a-2 (433, P)
BdorOR82a (399, F)
BdorOR45a (103;85, P)
BdorOR67d-3-v2 (230, P)
BdorOR67d-3-v1 (228, P)
BdorOr67d-2 (402, P)
BdorOR67d-4 (257, P)
BdorOR67d-1 (388, F)
BdorOR94b-1 (396, F)
BdorOR10a (307, P)
BdorOR24a (135;70, P)
BdorOR47b (449, F)
BdorOR94b-2 (402, F)
BdorOR13a (441, F)
BdorOR74a (414, F)
BdorOR35a (417, F)
BdorOR49a (179, P)
BdorORCO (473, F)
BdorOR67c-v2 (230, P)
BdorOR67c-v1 (405, F)
BdorOR88a (411, P)
BdorOR92a (325, P)
BdorOR69a-2 (180, P)
BdorOR69a-1 (189, P)
BdorOR63a-2-v1 (417, F)
BdorOR63a-1-v1 (83;343, P)
BdorOR63a-3 (411, P)
BdorOR49b-2-v2 (288, P)
BdorOR49b-2-v1 (287, P)
BdorOR49b-1 (257, P)
BdorOR43a-1 (364, P)
BdorOR43a-2-v1 (375, F)
BdorOR30a (237, P)
BdorOR43a-2-v2 (250, P)
0.5

Fig. 2

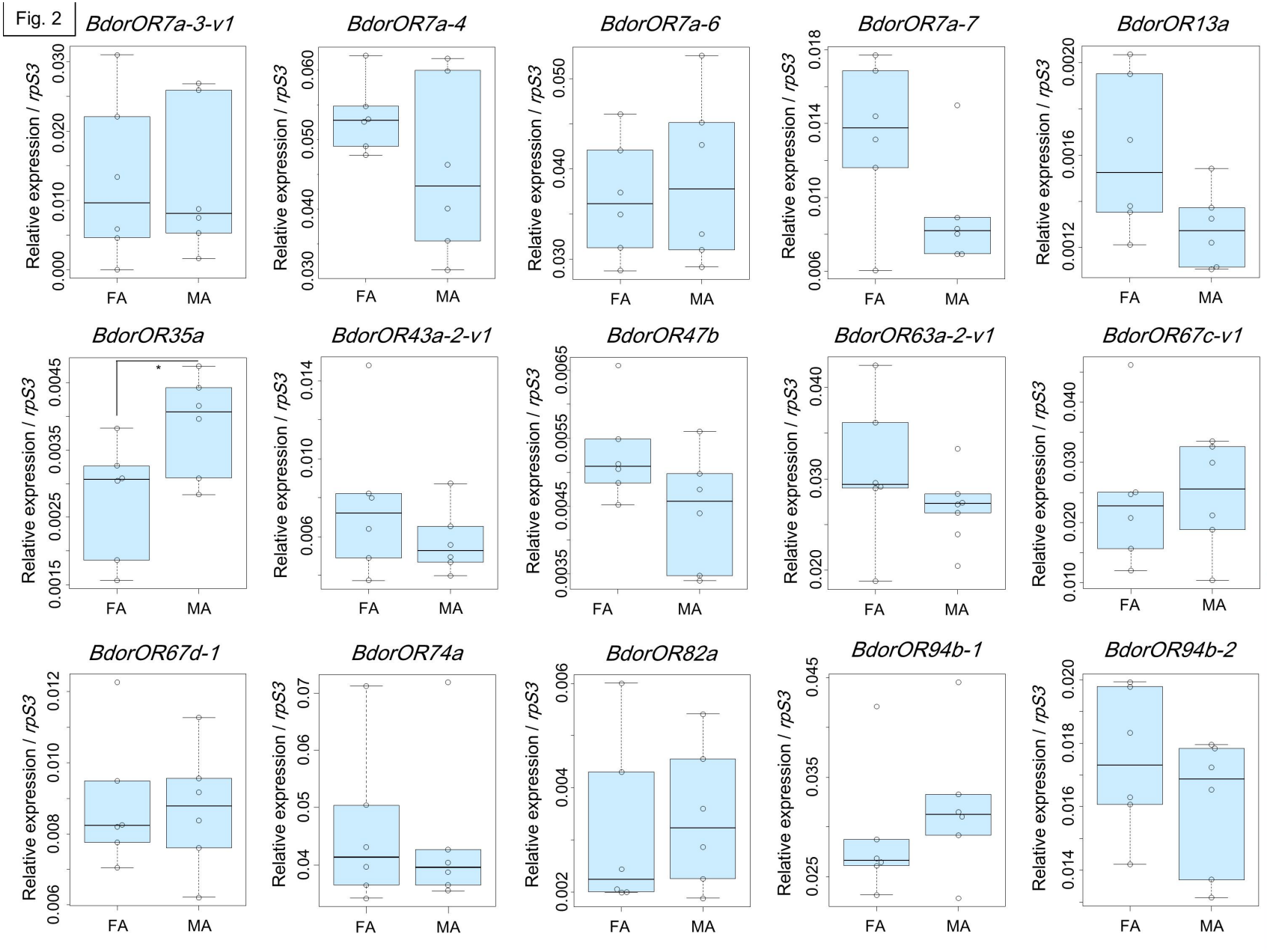


Fig. 3

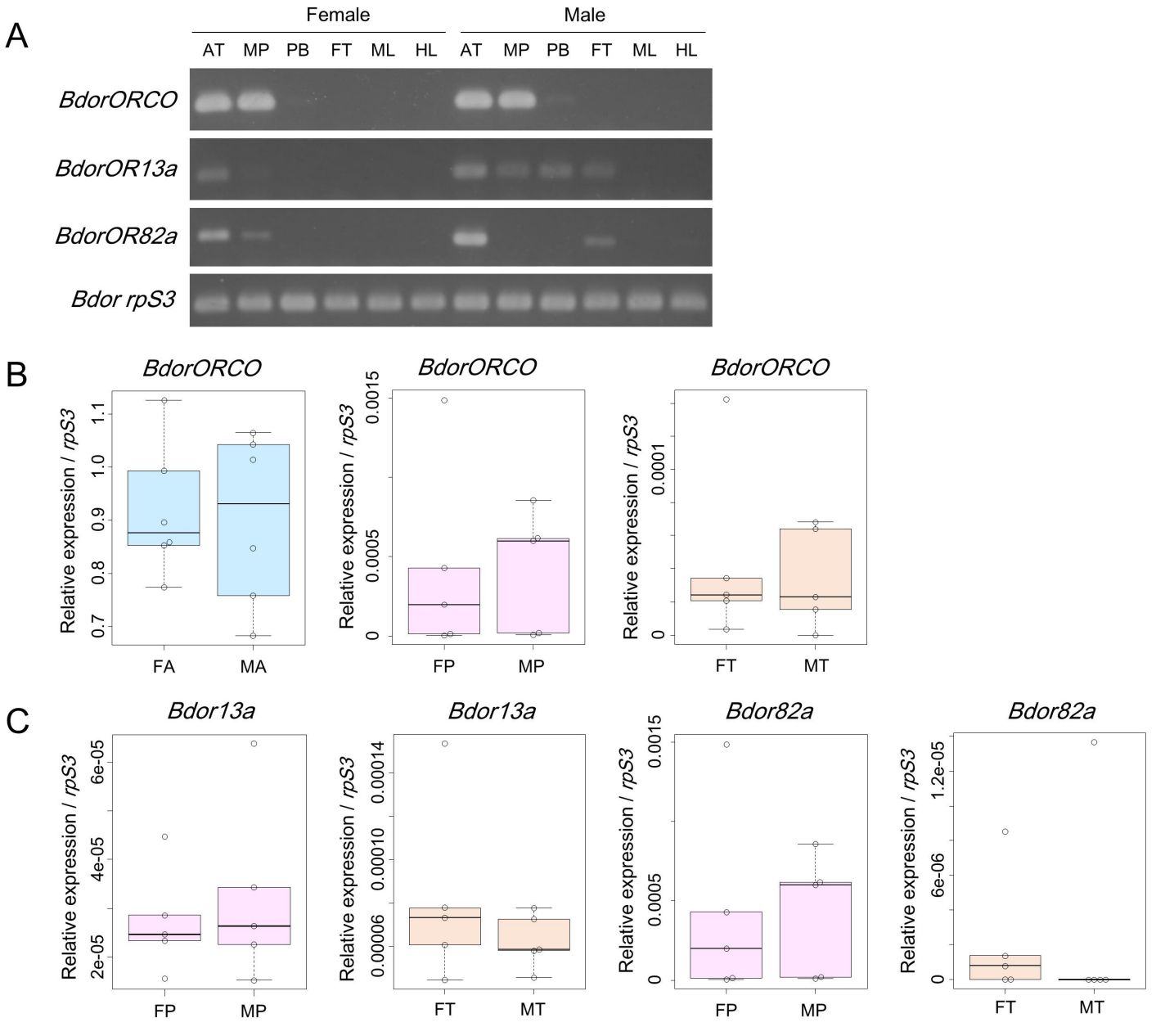


Fig. 4

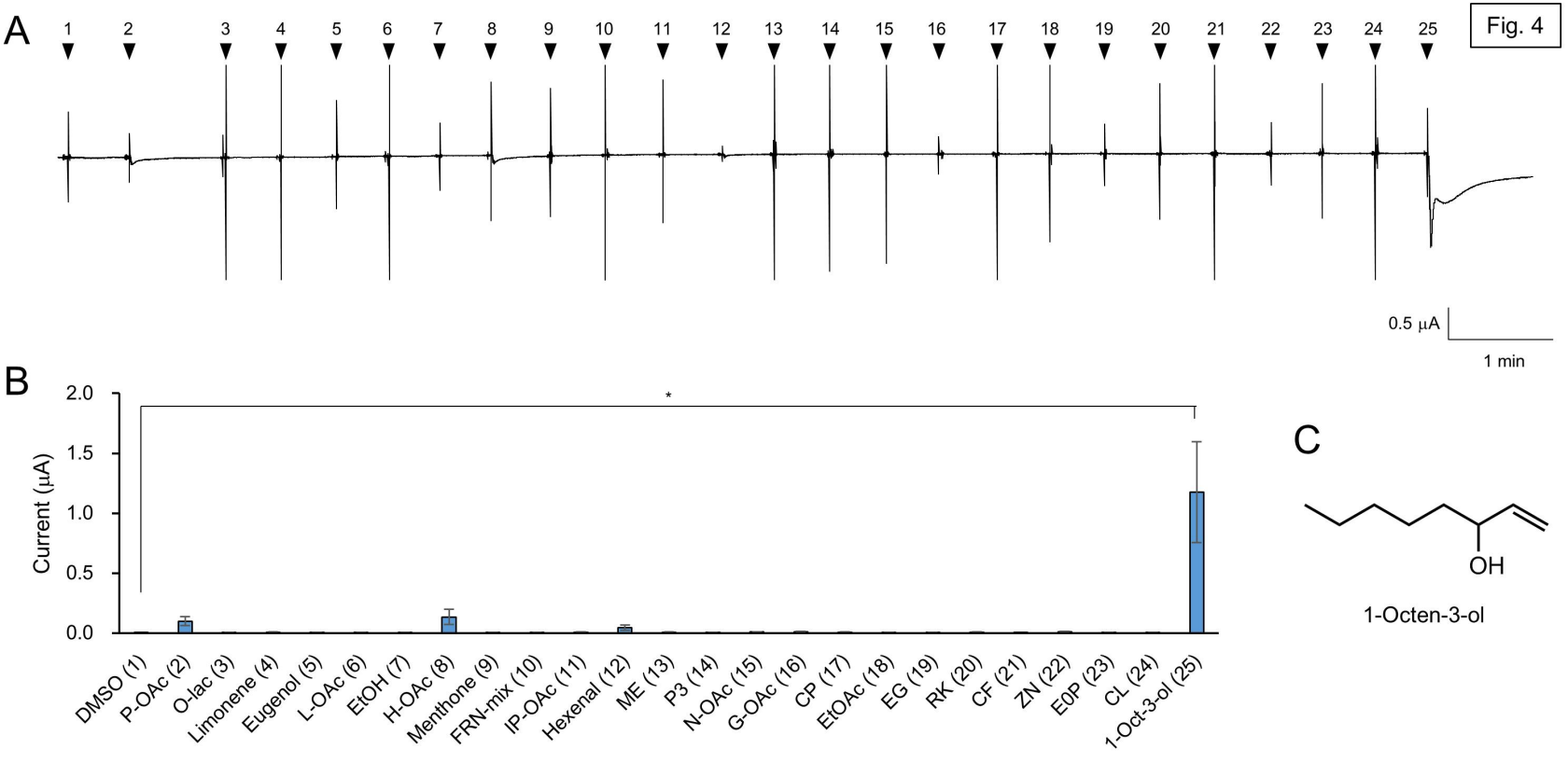
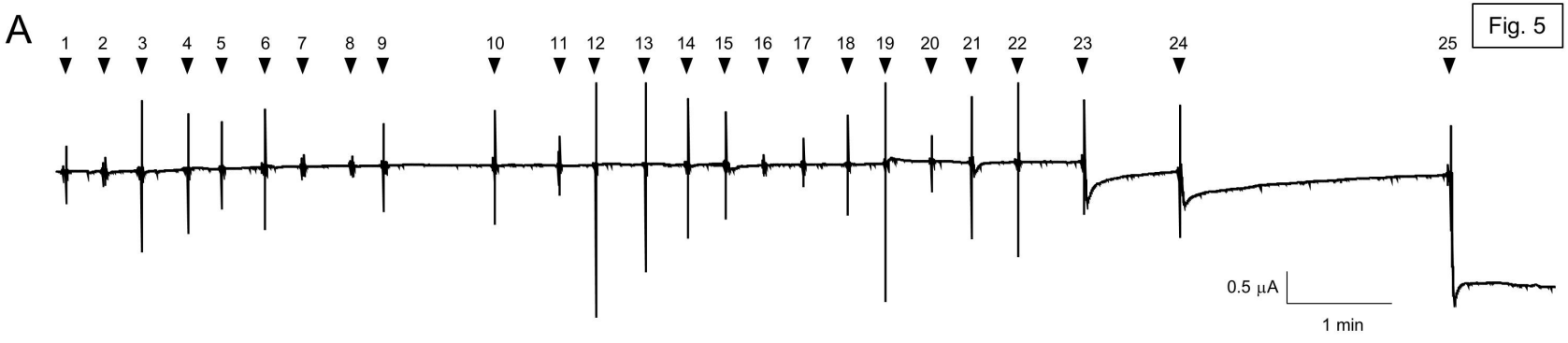
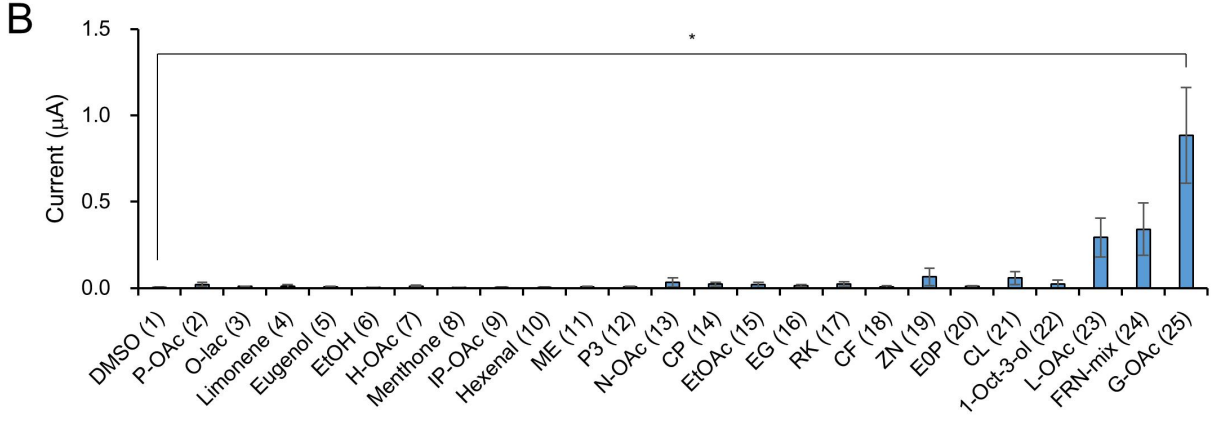
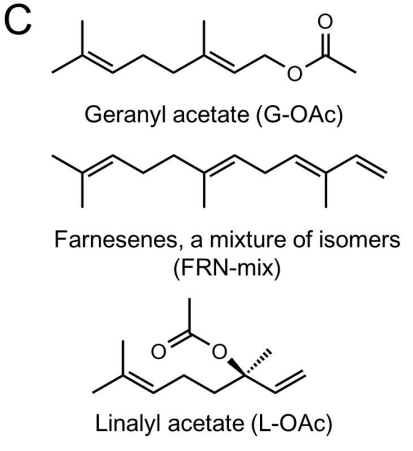
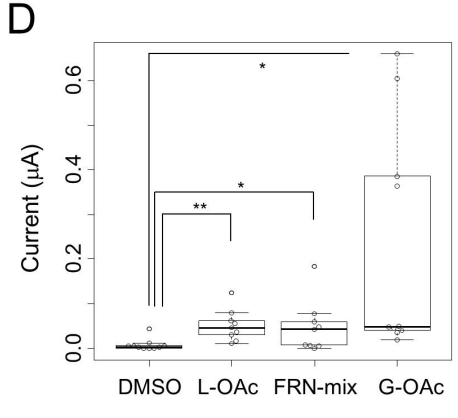


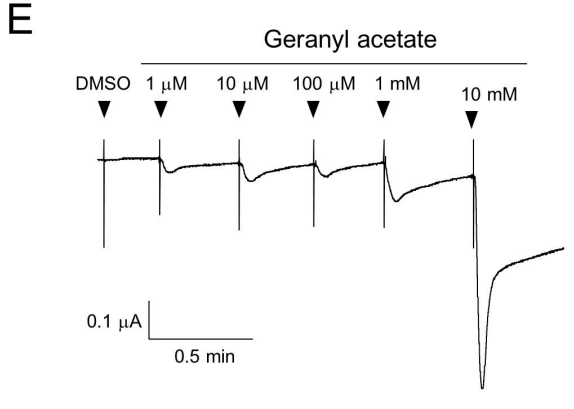
Fig. 5











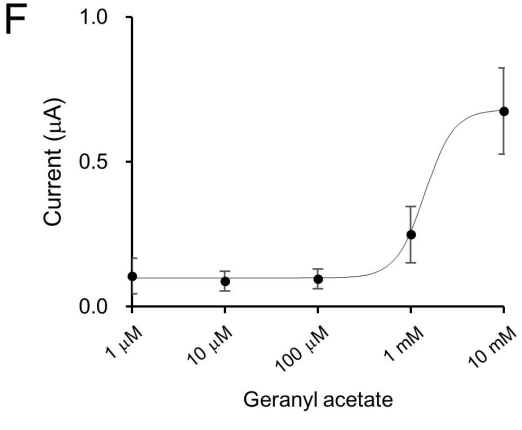


Fig. 6

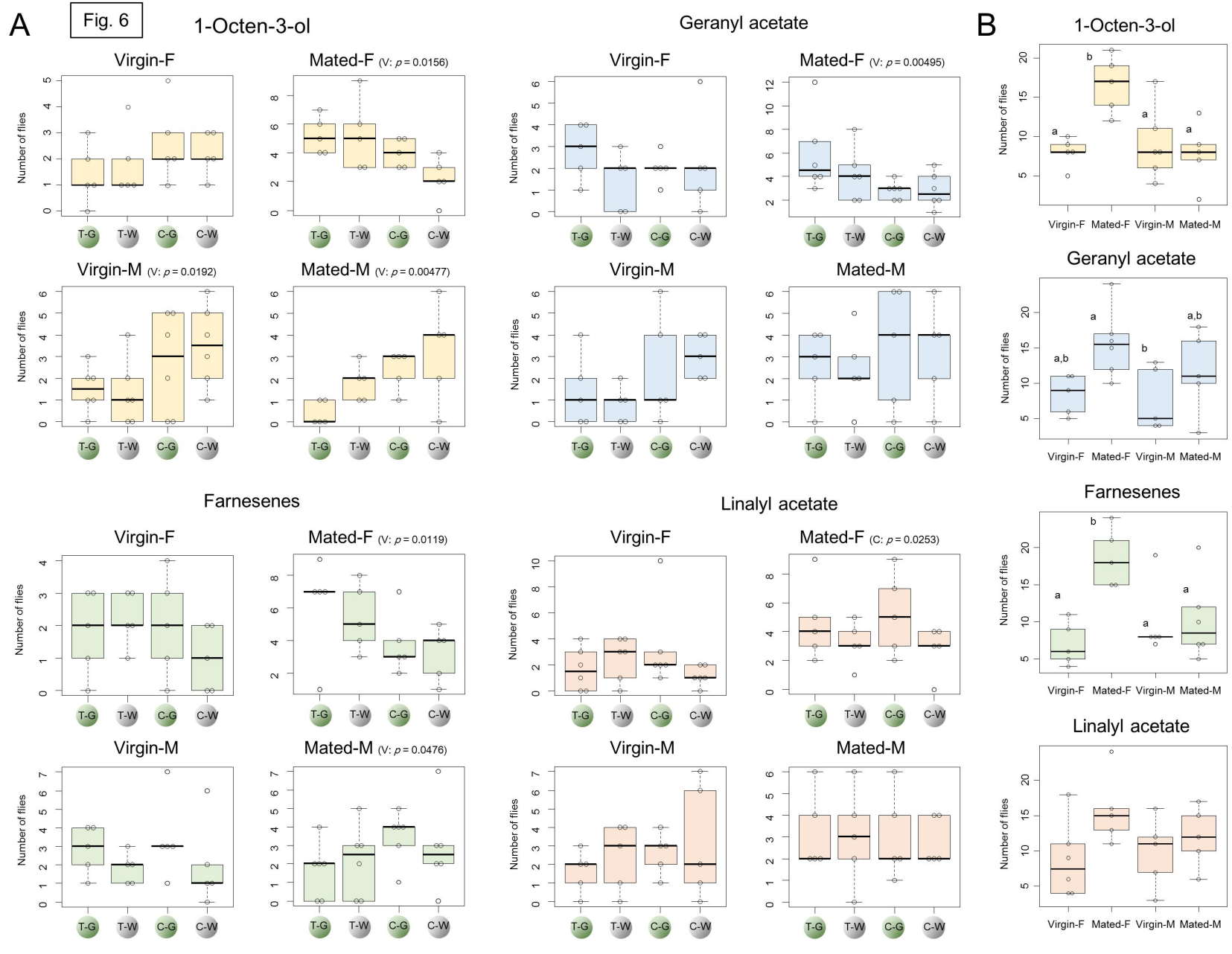


Table 1. Summary of sequence data analysis.

	Antenna	Proboscis
Number of raw reads	1,675,116	2,159,685
Number of clean reads	1,159,879	1,383,389
Number of assembled contigs	71,766	
Mean length of contigs (bp)	486	

Table 2. Candidate chemosensory receptor genes identified from the transcriptome.

Gene name	Length (AA)	CDS	E-value	BLASTP best hit (Accession number; Name; Species)	Reference[1] (Accession number or reference name)
ORs					
BdorORCO	473	Full	0	ADK97803.1; Or83b (ORCO) *Zeugodacus cucurbitae*	Ref. 1: KP743711; Ref. 2: CL538C2
BdorOR2a	93; 71	Partial	2e-61; 2e-41	XP_011198390.1; OR2a-like; *Bactrocera dorsalis*	
BdorOR7a-1	311	Partial	0	XP_019847175.1; OR7a-like; *Bactrocera dorsalis*	Ref. 1: KP743712; Ref. 2: U14846
BdorOR7a-2	433	Partial	0	XP_019847361.1; OR7a-like; *Bactrocera dorsalis*	Ref. 1: KP743713; Ref. 2: U16745
BdorOR7a-3-v1	398	Full	0	AKI29030.1; OR7a-3; *Bactrocera dorsalis*	Ref. 1: KP743714
BdorOR7a-3-v2	351	Partial	0	AKI29030.1; OR7a-3; *Bactrocera dorsalis*	
BdorOR7a-4	394	Full	0	XP_011198720.1; OR7a-like; *Bactrocera dorsalis*	Ref. 1: KP743715; Ref. 2: U15921
BdorOR7a-5-v1	400	Partial	0	XP_019845111.1; OR7a-like; *Bactrocera dorsalis*	Ref. 1: KP743716; Ref. 2: C2326C1
BdorOR7a-5-v2	398	Partial	0	XP_019845111.1; OR7a-like; *Bactrocera dorsalis*	
BdorOR7a-5-v3	370	Partial	0	XP_019845111.1; OR7a-like; *Bactrocera dorsalis*	
BdorOR7a-5-v4	368	Partial	0	XP_019845111.1; OR7a-like; *Bactrocera dorsalis*	
BdorOR7a-6	399	Full	0	XP_011208901.1; OR59b-like; *Bactrocera dorsalis*	Ref. 2: C1686C3
BdorOR7a-7	400	Full	0	XP_019846037.1; OR7a-like; *Bactrocera dorsalis*	Ref. 1: KP743721; Ref.2: C3971C1
BdorOR7a-8	238; 112	Partial	2e-156; 5e-69	XP_018798519.1; OR59b-like; *Bactrocera latifrons*	Ref. 2: C788C3
BdorOR7a-9	250	Partial	3e-106	XP_019845111.1; OR7a-like; *Bactrocera dorsalis*	Ref. 2: C2460C2
BdorOR7a-10	188	Partial	4e-130	AKI29029.1; OR7a-2; *Bactrocera cucurbitae*	Ref. 2: U4218
BdorOR7a-11	90	Partial	2e-56	XP_019846037.1; OR7a-like; *Bactrocera dorsalis*	
BdorOR10a	307	Partial	0	XP_018791769.1; OR10a; *Bactrocera latifrons*	Ref. 2: C4154C1
BdorOR13a	441	Full	0	AKI29033.1; OR13a; *Bactrocera dorsalis*	Ref. 1: KP743717; Ref. 2: C739C1
BdorOR24a	135; 70	Partial	9e-91; 4e-40	XP_011199522.1; OR24a; *Bactrocera dorsalis*	
BdorOR30a	237	Partial	5e-128	XP_019847596.1; OR30a-like; *Bactrocera dorsalis*	
BdorOR35a	417	Full	0	XP_019844437.1; OR35a-like; *Bactrocera dorsalis*	Ref. 1: KP743718; Ref. 2: U10148
BdorOR42b	228	Partial	4e-151	XP_011210512.1; OR7a-like; *Bactrocera dorsalis*	Ref. 2: U4218
BdorOR43a-1	364	Partial	0	AKI29035.1; OR43a-1; *Bactrocera dorsalis*	Ref. 1: KP743719

BdorOR43a-2-v1	375	Full	0	AKI29036.1; OR43a-2; *Bactrocera dorsalis*	Ref. 1: KP743720
BdorOR43a-2-v2	250	Partial	4e-179	XP_019847608.1; Or2-like; *Bactrocera dorsalis*	Ref. 2: C3544C1
BdorOR43a-3	72	Partial	1e-37	XP_014097484.1; Or2-like; *Bactrocera oleae*	
BdorOR45a	103; 85	Partial	8e-66; 6e-54	XP_011212447.2; OR45a-like; *Bactrocera dorsalis*	
BdorOR47b	449	Full	0	XP_019847427.1; OR47b; *Bactrocera dorsalis*	
BdorOR49a	179	Partial	1e-126	XP_011212431.1; OR49a-like; *Bactrocera dorsalis*	Ref. 2: U11993
BdorOR49b-1	257	Partial	0	XP_019845516.1; OR49b; *Bactrocera dorsalis*	Ref. 1: KP743723; Ref. 2: C6087C2
BdorOR49b-2-v1	287	Partial	2e-170	XP_019847679.1; OR49b-like; *Bactrocera dorsalis*	
BdorOR49b-2-v2	288	Partial	0	XP_019847679.1; OR49b-like; *Bactrocera dorsalis*	Ref. 1: KP743724
BdorOR49b-3	105	Partial	2e-67	AKI29039.1; OR49b-1; *Bactrocera dorsalis*	
BdorOR49b-4	83	Partial	4e-50	XP_019847607.1; OR2-like; *Bactrocera dorsalis*	
BdorOR59a	387	Partial	0	AKI29041.1; OR59a; *Bactrocera dorsalis*	Ref. 1: KP743725; Ref. 2: U350
BdorOR63a-1-v1	83; 343	Partial	1e-49; 0	AKI29042.1; OR63a-1; *Bactrocera dorsalis*	Ref. 1: KP743726
BdorOR63a-1-v2	52	Partial	2e-21	AKI29042.1; OR63a-1; *Bactrocera dorsalis*	
BdorOR63a-1-v3	82	Partial	6e-48	XP_018787905.1; OR63a-like; *Bactrocera latifrons*	
BdorOR63a-2-v1	417	Full	0	AKI29043.1; OR63a-2; *Bactrocera dorsalis*	Ref. 1: KP743727; Ref. 2: U11167
BdorOR63a-2-v2	139	Partial	4e-70	XP_019847162.1; OR63a-like; *Bactrocera dorsalis*	
BdorOR63a-3	411	Partial	0	XP_018783180.1; OR63a; *Bactrocera latifrons*	Ref. 2: U1859
BdorOR67c-v1	405	Full	0	XP_011200400.1; OR67c-like; *Bactrocera dorsalis*	Ref. 1: KP743728; Ref. 2: C173C1
BdorOR67c-v2	230	Partial	2E-152	XP_011200401.1; OR67c-like; *Bactrocera dorsalis*	
BdorOR67d-1	388	Full	0	XP_011203703.1; OR67d-like; *Bactrocera dorsalis*	Ref. 1: KP743729; Ref. 2: C8295C1
BdorOR67d-2	402	Partial	4e-178	XP_017473047.1; OR67d-like; *Rhagoletis zephyria*	
BdorOR67d-3-v1	228	Partial	2e-133	XP_017473047.1; OR67d-like; *Rhagoletis zephyria*	Ref. 2: U33
BdorOR67d-3-v2	230	Partial	6e-138	XP_017473047.1; OR67d-like; *Rhagoletis zephyria*	
BdorOR67d-4	257	Partial	0	XP_011203704.2; OR67d-like; *Bactrocera dorsalis*	Ref. 2: U3061
BdorOR69a-1	189	Partial	3e-133	AKI29046.1; OR69a; *Bactrocera dorsalis*	Ref. 1: KP743730
BdorOR69a-2	180	Partial	3e-103	XP_011191113.1; OR69a isoformA; *Bactrocera cucurbitae*	Ref. 2: U12022
BdorOR69a-3	69	Partial	5e-42	XP_011209369.1; putative OR69a; *Bactrocera dorsalis*	
BdorOR74a	414	Full	0	XP_011201924.2; OR74a-like; *Bactrocera dorsalis*	Ref. 1: KP743731

BdorOR82a	399	Full	0	XP_011208732.1; OR82a; *Bactrocera dorsalis*	Ref. 2: U803
BdorOR83a	47	Partial	3e-16	XP_011184142.1; OR83a-like; *Zeugodacus cucurbitae*	
BdorOR85d	85	Partial	3e-37	XP_018801738.1; OR85d; *Bactrocera latifrons*	
BdorOR88a	411	Partial	0	AKI29048.1; OR88a; *Bactrocera dorsalis*	Ref. 1: KP743732; Ref. 2: 5300C1
BdorOR92a	325	Partial	0	XP_011208819.1; OR92a; *Bactrocera dorsalis*	
BdorOR94b-1	396	Full	0	XP_019847876.1; OR94b-like; *Bactrocera dorsalis*	Ref. 1: KP743733; Ref. 2: U3077
BdorOR94b-2	402	Full	0	XP_018801531.1; OR94b-like; *Bactrocera latifrons*	Ref. 2: U3948
GRs					
BdorGR5a	79; 100	Partial	3e-47; 2e-63	XP_011213356.2; GR5a; *Bactrocera dorsalis*	
BdorGR8a	75; 43	Partial	8e-41; 0.014	XP_011185249.1; GR8a-like; *Zeugodacus cucurbitae*	
BdorGR21a-1	456	Full	0	XP_011204023.1; GR21a; *Bactrocera dorsalis*	Ref. 1: KP743664; Ref. 2; U13527
BdorGR21a-2-v1	432	Partial	0	AOE48126.1; GR6; *Scaeva pyrastri*	
BdorGR21a-2-v2	424	Partial	0	AOE48126.1; GR6; *Scaeva pyrastri*	
BdorGR21a-2-v3	339	Partial	7e-136	AOE48126.1; GR6; *Scaeva pyrastri*	
BdorGR21a-2-v4	184	Partial	2e-62	AOE48126.1; GR6; *Scaeva pyrastri*	
BdorGR28b	110	Partial	8e-69	XP_011180327.1; putative GR28b; *Zeugodacus cucurbitae*	
BdorGR32a-1	353	Partial	0	XP_019847005.1; GR32a; *Bactrocera dorsalis*	Ref. 1: KP743666
BdorGR32a-2	76	Partial	1e-40	XP_018792133.1; uncharacterized protein LOC108970891; *Bactrocera latifrons*	
BdorGR39b	106	Partial	3e-46	XP_004536482.1; GR39b; *Ceratitis capitata*	
BdorGR43a	67; 75	Partial	6e-38; 3e-43	XP_019845196.1; GR43a-like; *Bactrocera dorsalis*	
BdorGR63a	485	Full	0	XP_011212836.1; GR63a; *Bactrocera dorsalis*	Ref. 1: KP743667
BdorGR64b	67	Partial	3e-39	XP_011213352.1; GR64b; *Bactrocera dorsalis*	
BdorGR64e	59; 71; 62	Partial	2e-32; 3e-42; 3e-26	XP_011213347.1; GR64e; *Bactrocera dorsalis*	
BdorGR64f	278	Partial	5e-147	XP_018783853.1; uncharacterized protein LOC108965721; *Bactrocera latifrons*	
BdorGR98b	69	Partial	7e-42	XP_011205406.1; putative GR98b; *Bactrocera dorsalis*	
IRs					
BdorIR8a	104; 673	Partial	1e-54; 0	XP_011211753.1; glutamate receptor ionotropic, kainate 2; *Bactrocera dorsalis*	Ref. 2: C8433C2
BdorIR8a-2	153	Partial	5e-100	XP_014100759.1; glutamate receptor ionotropic, kainate 2-like; *Bactrocera oleae*	

BdorIR25a	940	Full	0	XP_011207795.1; IR25a; *Bactrocera dorsalis*	Ref. 1: U215
BdorIR31a-1	108	Partial	1e-54	XP_018804290.1, uncharacterized protein LOC108978446, *Bactrocera latifrons*	
BdorIR31a-2	83	Partial	1e-27	XP_012162538.1; LOC101456253, *Ceratitis capitata*	
BdorIR40a	128; 265; 83	Partial	6e-85; 0; 1e-47	XP_011212457.2; uncharacterized protein LOC105232474; *Bactrocera dorsalis*	Ref. 1: KP743669; Ref. 2: U9427
BdorIR41a	135; 87; 213	Partial	5e-91; 1e-48; 8e-147	AKI28986.1; IR41a; *Bactrocera dorsalis*	Ref. 1: KP743670
BdorIR56c	118; 216	Partial	1e-12; 4e-132	XP_018794909.1; uncharacterized protein LOC108972669; *Bactrocera latifrons*	
BdorIR64a-1	322	Partial	0	XP_018799073.1; uncharacterized protein LOC108975188; *Bactrocera latifrons*	Ref. 2: U7132
BdorIR64a-2	96	Partial	1e-58	XP_019845172.1; uncharacterized protein LOC105224490; *Bactrocera dorsalis*	
BdorIR75a-1	342	Partial	0	XP_019845038.1; uncharacterized protein LOC109579404; *Bactrocera dorsalis*	Ref. 2: U14774
BdorIR75a-2	140; 232	Partial	2e-79; 2e-164	XP_019845037.1; glutamate receptor; *Bactrocera dorsalis*	
BdorIR75b	95	Partial	2e-49	XP_014088428.1; uncharacterized protein LOC106616338; *Bactrocera oleae*	
BdorIR75d	162; 105;	Partial	5e-110; 3e-64; 0	XP_019844868.1; uncharacterized protein LOC105223467; *Bactrocera dorsalis*	Ref. 1: KP743671
BdorIR76a-1	137; 147	Partial	7e-92; 3e-100	XP_011204763.1; uncharacterized protein LOC105227219; *Bactrocera dorsalis*	
BdorIR76a-2	286	Partial	0	XP_014086277.1; uncharacterized protein LOC106614874; *Bactrocera oleae*	
BdorIR76b	659	Full	0	AKI28988.1; IR76b; *Bactrocera dorsalis*	Ref. 1: KP743672; Ref. 2: C1154C3
BdorIR84a	703	Partial	0	XP_011193628.1; glutamate receptor 1; *Zeugodacus cucurbitae*	Ref. 1: KP743673
BdorIR92a-1	140; 116; 246	Partial	3e-85; 4e-53; 4e-177	AKI28990.1; IR92a; *Bactrocera dorsalis*	Ref. 1: KP743674; Ref. 2: C2923C2
BdorIR92a-2	146	Partial	2e-92	XP_019845172.1; uncharacterized protein LOC105224490; *Bactrocera dorsalis*	
BdorIR93a-1	93; 602	Partial	7e-55; 0	XP_011214752.1; glutamate receptor ionotropic, delta-1; *Bactrocera dorsalis*	Ref. 2: U7132
BdorIR93a-2	76	Partial	1e-38	XP_014095980.1; uncharacterized protein LOC106621575; *Bactrocera oleae*	
BdorIR94f	95	Partial	3e-58	XP_011199185.1; uncharacterized protein LOC105223232; *Bactrocera dorsalis*	

[1]Homologs representing more than 90% amino acid identities with chemosensory receptors identified in the present study are listed as references. Ref. 1: Wu et al., 2015; Ref.2: Liu et al., 2016.

Table 3. Results of data analysis using a generalized linear model with a binomial logit fit to identify the factors that influenced the numbers of fruit flies landing on the spheres.

Volatile	Fruit fly	Factor	AIC	Deviance	*P*
1-Octen-3-ol	Virgin female	Null	64.9	–	–
		Volatile (V)	65.2	1.68	0.195
		Color (C)	66.9	0.00	1.000
		V × C	67.2	1.68	0.432
	Mated female	Null	85.0	–	–
		Volatile (V)	81.2	5.85	0.016*
		Color (C)	86.0	1.07	0.302
		V × C	82.1	6.92	0.031*
	Virgin male	Null	79.3	–	–
		Volatile (V)	75.8	5.48	0.019*
		Color (C)	81.3	0.00	1.000
		V × C	77.8	5.48	0.065
	Mated male	Null	73.9	–	–
		Volatile (V)	67.9	7.97	0.005**
		Color (C)	72.6	3.27	0.071
		V × C	66.6	11.26	0.004**
Geranyl acetate	Virgin female	Null	71.4	–	–
		Volatile (V)	73.4	0.00	1.000
		Color (C)	72.5	0.90	0.343
		V × C	74.5	0.90	0.638
	Mated female	Null	105.6	–	–
		Volatile (V)	99.7	7.90	0.005**
		Color (C)	106.4	1.16	0.282
		V × C	100.5	9.06	0.011*
	Virgin male	Null	77.4	–	–
		Volatile (V)	72.1	7.22	0.007**
		Color (C)	79.4	0.00	1.000
		V × C	74.1	7.22	0.027*
	Mated male	Null	88.5	–	–
		Volatile (V)	89.4	1.17	0.278
		Color (C)	90.5	0.07	0.787
		V × C	91.3	1.25	0.536
Farnesenes	Virgin female	Null	65.3	–	–

		Volatile (V)	66.5	0.74	0.389
		Color (C)	67.0	0.27	0.606
		V × C	68.3	1.01	0.604
	Mated female	Null	92.7	–	–
		Volatile (V)	88.4	6.33	0.012*
		Color (C)	94.2	0.58	0.446
		V × C	89.8	6.92	0.031*
	Virgin male	Null	77.0	–	–
		Volatile (V)	78.6	0.34	0.562
		Color (C)	75.9	3.06	0.080
		V × C	77.6	3.40	0.183
	Mated male	Null	98.4	–	–
		Volatile (V)	96.5	3.92	0.048*
		Color (C)	100.4	0.02	0.895
		V × C	98.5	3.94	0.139
Linalyl acetate	Virgin female	Null	98.6	–	–
		Volatile (V)	100.5	0.08	0.777
		Color (C)	99.3	1.29	0.256
		V × C	101.3	1.37	0.504
	Mated female	Null	90.2	–	–
		Volatile (V)	92.2	0.01	0.907
		Color (C)	87.2	5.01	0.025*
		V × C	89.2	5.02	0.081
	Virgin male	Null	81.8	–	–
		Volatile (V)	82.0	1.75	0.186
		Color (C)	82.7	1.06	0.304
		V × C	83.0	2.80	0.246
	Mated male	Null	77.4	–	–
		Volatile (V)	79.3	0.07	0.790
		Color (C)	79.3	0.07	0.790
		V × C	81.3	0.14	0.932

*$p < 0.05$, **$p < 0.01$.

Supplemental figure captions

Fig. S1. Chemical structures of tested compounds for the functional analysis of candidate olfactory receptors (ORs).

Fig. S2. Behavioral bioassay to evaluate the attractiveness of the volatiles. (A) The meshed cage (40 cm × 40 cm × 40 cm) used for the behavioral bioassay. We placed one sphere 10 cm from each corner of the cage. (B) To test each compound, we impregnated a piece of filter paper (15 mm × 3 mm) with 1 mg of the compound dissolved in 5 μL of ethanol, and dried the paper at room temperature. Each filter paper was then placed in a clean 0.2-mL clear microtube, which was positioned facing up in one of the holes of a polyethylene sphere that consisted of 26 holes. Each sphere was placed on a plastic petri dish (diameter 5 cm) to prevent rolling on the cage floor during the bioassay.

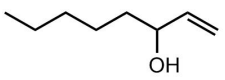
Fig. S3. Comparison of amino acid sequences of olfactory receptors (ORs). Alignments of BdorOR13a and DmOR13a (A), and BdorOR82a and DmOR82a (B) are shown. Seven transmembrane domains (TM1–TM7) are indicated by solid lines.

Fig. S4. Effects of ethanol and visual cues on the landing behavior of mated *Bactrocera dorsalis* females. The box plot shows 25–75% (box), median (band inside), and minima to maxima (whiskers). (A) Numbers of mated females landing on the green or white spheres. The numbers of flies are plotted as dots (n = 7). T-G, T-W, C-G, and C-W indicate volatile-treated green balls, volatile-treated white balls, control (volatile-untreated) green balls, and control white balls, respectively. The

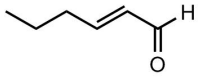
table shows the results of data analysis using a generalized linear model with a binomial logit fit to identify the factors that influenced the number of fruit flies landing on the spheres. Neither the volatile nor the color of the spheres had a significant effect (p-values > 0.05). (B) Comparison of the total numbers of mated females landing on the spheres calculated from Fig. 6A and S4A. We detected no significant differences between the treatments at $p < 0.05$ according to Tukey's HSD test.

Fig. S1

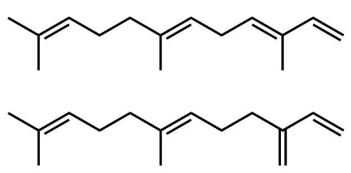
Ethanol (EtOH)



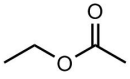
(±)-1-Octen-3-ol (1-Oct-3-ol)



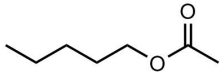
(*E*)-2-Hexenal (Hexenal)



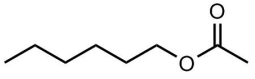
Farnesenes, mixture of isomers (FRN-mix)



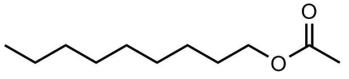
Ethyl acetate (EtOAc)



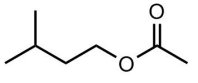
Pentyl acetate (P-OAc)



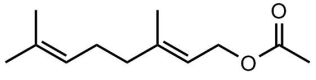
Hexyl acetate (H-OAc)



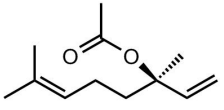
Nonyl acetate (N-OAc)



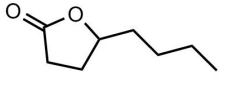
Isopentyl acetate (IP-OAc)



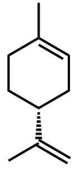
Geranyl acetate (G-OAc)



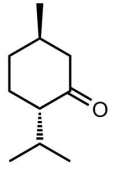
Linalyl acetate (L-OAc)



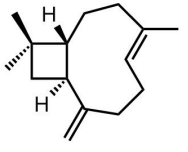
(±)-γ-Octalactone (O-lac)



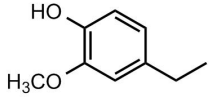
(*R*)-(+)-Limonene (Limonene)



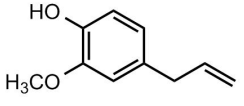
(−)-Menthone (Menthone)



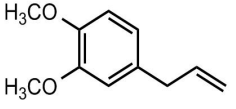
β-Caryophyllene (CP)



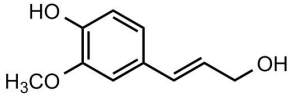
4-Ethylguaiacol (EG)



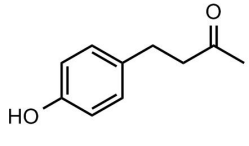
Eugenol



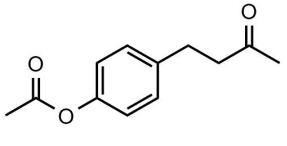
Methyl eugenol (ME)



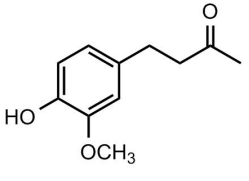
(*E*)-Coniferyl alcohol (CF)



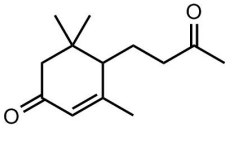
Raspberry ketone (RK)



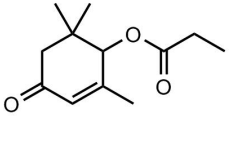
Cue-lure (CL)



Zingerone (ZN)



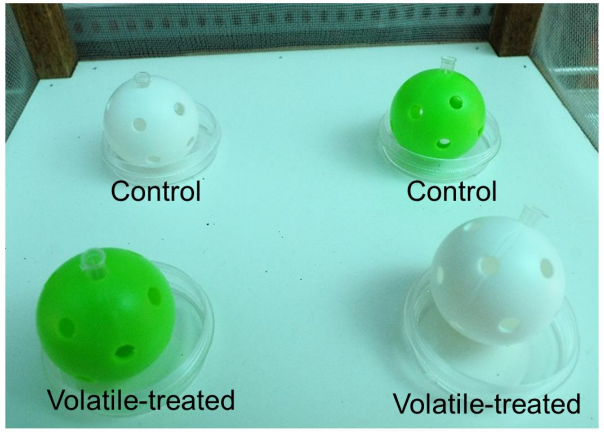
(±)-3-Oxo-7,8-dihydro-α-ionone (P3)



(±)-4-Propionyloxyisophorone (E0P)

Fig. S2

A



B

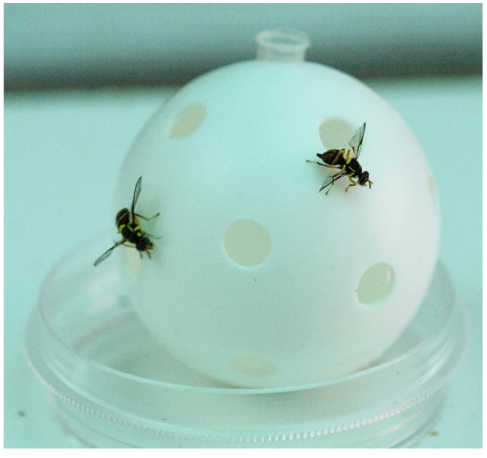
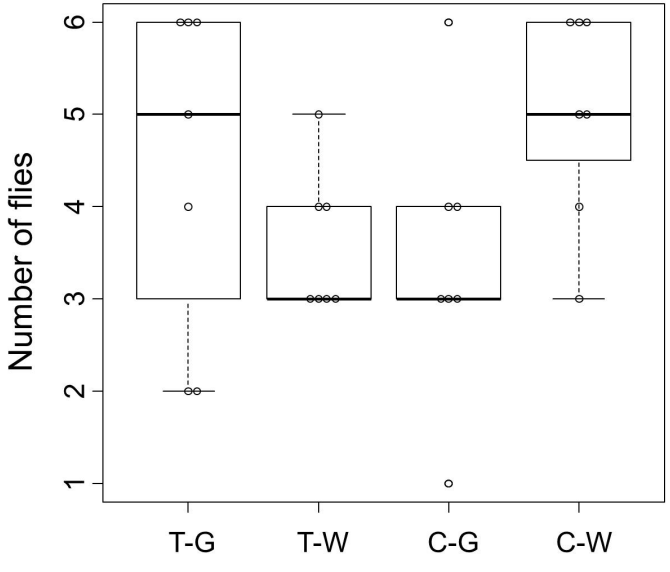


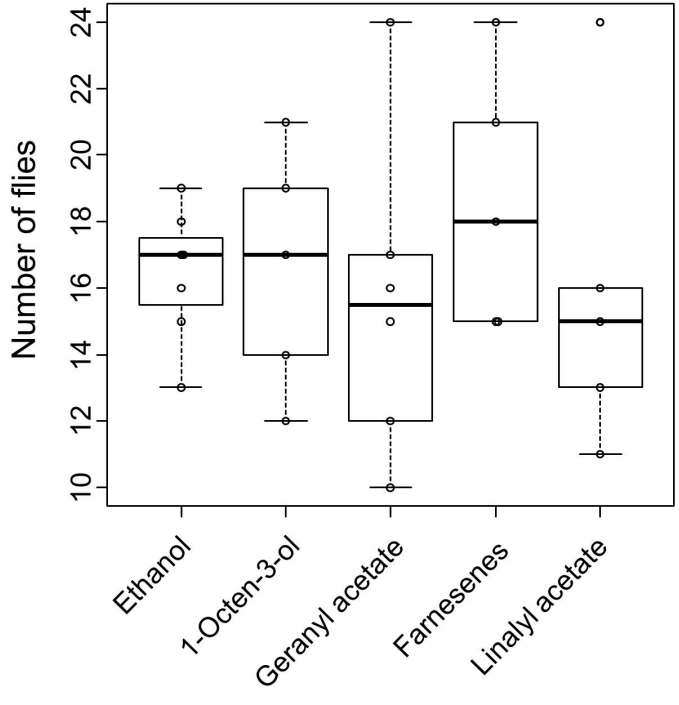
Fig. S3

Fig. S4

A



B



Volatile	Factor	AIC	Deviance	*P*
Ethanol	Null	105.9	-	-
	Volatile (V)	107.8	0.0853	0.770
	Color (C)	107.7	0.237	0.626
	V X C	109.6	0.322	0.851